UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

TESIS DOCTORAL

Filogenia, filogeografía y evolución de *Luciobarbus* Heckel, 1843 (Actinopterygii, Cyprinidae) en la cuenca del Mediterráneo occidental

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Director

Ignacio Doadrio Villarejo

Madrid, 2017

PHYLOGENY, PHYLOGEOGRAPHY AND EVOLUTION OF LUCIOBARBUS HECKEL, 1843 IN THE WESTERN MEDITERRANEAN

Luciobarbus sclateri (Günther, 1868)
-Illustration by Filipa Filipe-

MIRIAM CASAL LÓPEZ

2017

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Facultad de Ciencias Biológicas

Departamento de Zoología y Antropología física

Phylogeny, phylogeography and evolution of *Luciobarbus* Heckel, 1843, in the western Mediterranean

Memoria presentada para optar al grado de Doctor por

Miriam Casal López

Bajo la dirección del Doctor

Ignacio Doadrio Villarejo

Madrid - Febrero 2017

Ignacio Doadrio Villarejo, Científico Titular del Museo Nacional de Ciencias Naturales – CSIC

CERTIFICAN:

Que la presente memoria titulada ”Phylogeny, phylogeography and evolution of *Luciobarbus* Heckel, 1843, in the western Mediterranean” que para optar al grado de Doctor presenta Miriam Casal López, ha sido realizada bajo mi dirección en el Departamento de Biodiversidad y Biología Evolutiva del Museo Nacional de Ciencias Naturales – CSIC (Madrid). Esta memoria está además adscrita académicamente al Departamento de Zoología y Antropología Física de la Facultad de Ciencias Biológicas de la Universidad Complutense de Madrid. Considerando que representa trabajo suficiente para constituir una Tesis Doctoral, autorizamos su presentación.

Y para que así conste, firmamos el presente certificado,

El director:

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En Madrid, a XX de Febrero de 2017

El trabajo de esta Tesis Doctoral ha podido llevarse a cabo con la financiación de los proyectos del Ministerio de Ciencia e Innovación. Además, Miriam Casal López ha contado con una beca del Ministerio de Ciencia e Innovación.

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Ackowledgments/*Agradecimientos*

SUMMARY

This dissertation is composed of 5 chapters which try to assess the evolutionary patterns of the genus *Luciobarbus* Heckel, 1843 in the western Palearctic at different scales: from taxonomic remarks that have led to the description of new species, to the study of the phylogenetic relationships and the biogeographical patterns shaping the diversification and evolution processes that gave origin to the species currently inhabiting the Palearctic region with an special emphasis in the western Mediterranean. In chapters I and II, five new species from North Africa are being described based on morphologic and molecular characters; chapter III deals with the influence of paleohidrogeological changes on the history and population structure of an endemism from the Iberian Peninsula*;* chapter IV focuses on the history and evolution of populations inhabiting both sides of the Strait of Gibraltar and chapter V addresses a thorough phylogenetic revision of the genus in its entire distribution range.

In this thesis, the integration of different types of molecular tools, phylogenetic analyses and biogeographical methodologies have provided a wide scope for understanding the patterns and factors underlying the evolutionary history of the genus *Luciobarbus.* A detailed summary of each of the chapters is described as follows:

Chapter I

The taxonomy of *Barbus* sensu stricto Cuvier and Cloquet, 1816, has been reviewed in the last years and as consequence Mediterranean species previously included in this genus are presently assigned to the genus *Luciobarbus* Heckel, 1843. The population of North African species of *Luciobarbus* shows high diversification in Morocco, but there have been few taxonomic studies focusing on these populations. Previous phylogenetic studies based on morphological characters and molecular

markers (isozymes and mitochondrial *cytb*) found that the populations belonging to north-western Morocco presented high levels of genetic differentiation compared to other populations of the genus, that were already assigned to separate species. The aim of this work was to describe those populations as distinct species based on morphological, meristic and genetic traits. For this purpose, a total of 200 specimens were morphological studied from Laou, Loukos, Hachef, Grou and Sebou basins. Twenty-three morphometric measurements and nine meristic variables were recorded and compared through different tests and diagnose osteological structures were investigated through computer tomography. Phylogenetic analyses of the entire mitochondrial *cytb* gene of each population under study were included. Both morphological and genetic approaches supported the distinction of three different new species: *Luciobarbus maghrebensis, Luciobarbus rabatensis* and *Luciobarbus rifensis.*

Chapter II

The genus *Luciobarbus* strictly occurs in freshwater systems and shows wide habitat versatility and is usually present in rivers of different topology, allowing the existence of limnetic and rheophilic species. In Morocco the rheophilic species of *Luciobarbus* have been traditionally assigned to *Luciobarbus nasus* (Günther, 1874) and *Luciobarbus magniatlantis* (Pellegrin, 1919), although this last species is considered by some authors as a junior synonym of *L.nasus.* These rheophilic species of *Luciobarbus* show a series of morphological characteristics that differ from other *Luciobarbus* species, placing them in an unresolved taxonomic position and they could not be assigned to the genus *Luciobarbus* when the genus was formally defined on the basis of osteological characters. Previous molecular studies placed *L. nasus* in the genus *Luciobarbus* along with other North African species. Therefore and for disentangling this puzzle the populations of the rheophilic barbs of the three basins in which they are currently present were studied. A total of 60 specimens were morphological studied from Tensift, Oum er Rbia and Moulouya basins.

Twenty-four morphometric measurements and ten meristic variables were counted. Osteological characteristic were investigated through computer tomography and phylogenetic analyses of the entire mitochondrial *cytb* gene from up-to-five individuals from each population under study were included. The three studied population from the Tensift, Oum er Rbia and Moulouya basins possessed molecular, osteological and morphometric traits that identified them as different species. Based on these evidences, two new species were described: *Luciobarbus guercifensis* and *Luciobarbus zayanensis.*

Chapter III

The genus *Luciobarbus* shows high levels of local endemicity across its distribution range and its species are genetically and morphologically well-differentiated both under sympatric and allopatric conditions. The species *Luciobarbus sclateri* (Günther, 1868) is an endemism of the Iberian Peninsula, confined to the mid-south basins of this region, including Guadalquivir, Guadiana, Segura and small southern basins. The Iberian Peninsula, at the western area of the Mediterranean Basin is considered a hotspot, well-known for its species richness and high levels of endemisms. Since Cenozoic, Iberia has undergone several geological and climatic changes such as the Alpine orogeny that led to the uplift of its major mountain chains; the Messinian Salinity Crisis, an event that almost dried the Mediterranean Sea; the reopening of the Strait of Gibraltar and the influence of the Last Glacial Maximum. This complex past has shaped the freshwater network of this region through time and since this species disperses only via freshwater, it is expected that its present-day distribution has been greatly influenced by hydrogeological changes. In this context, *Luciobarbus sclateri* represents an ideal biological model to describe the imprint of these historical processes on the population structure and diversification patterns of the species. A total of 505 individuals from 22 basins covering the entire distribution range of the species were analyzed through molecular markers. Three major lineages

were found and the mtDNA distribution patterns supported the isolation of the hydrographic basins as main factor driving intraspecific differentiation, followed by secondary contact, admixture and re-isolation processes that led to the present-day distribution of *Luciobarbus sclateri.*

Chapter IV

The history of the western Mediterranean region has been strongly influenced by tectonic dynamics that have alternately connected and separated Eurasian and African landmasses, allowing fauna exchange. The independent movement of the Alboran domain during the Alpine orogeny created the Betic-Rif arc that rims the Mediterranean Sea from southern Spain across the Gibraltar arc and into Morocco. Currently, *Luciobarbus* represents the only primary freshwater fish widely distributed in the Mediterranean Basin showing its highest diversity at the western Mediterranean. This chapter focuses on the comparative study of the phylogeography of the populations of the genus *Luciobarbus* on both sides of the Strait of Gibraltar. All known population comprised within the basins of the Betic-Rif arc were sampled. Time calibrated analysis and biogeographical reconstruction of the complete mitochondrial *cytb* and a portion of nuclear *actin II* from 323 specimens of 23 species from 45 basins show that the western lineages of *Luciobarbus* originated during the Messinian, one including most of North African and western Algerian species and a second one composed of all the Iberian species and one species from eastern Morocco and another form eastern Algerian basins. These two lineages apparently occurred at two independent episodes, one associated to the opening and closing of the Atlantic-Mediterranean gateways (~7-6 mya) and a second one with the end of the Messinian (~5.3 mya).

Chapter V

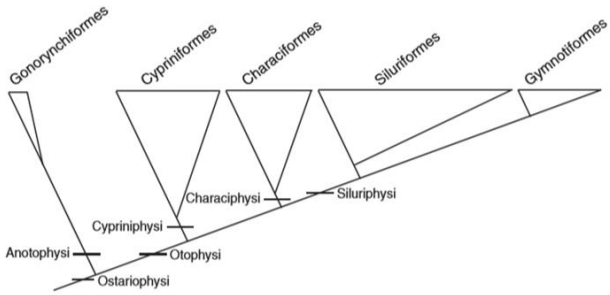
This chapter is focused on the evolutionary study of the genus *Luciobarbus* Heckel, 1843 through the most complete phylogeny carried out, so far. The genus comprises more than 30 species of medium to large size widely distributed in the Palearctic along the rivers draining into the Persian Gulf and the Mediterranean, Caspian and Black seas. This genus shows a high level of local endemism and restricted dispersal ability to freshwater ecosystems. Colonization of new areas through freshwater network and phylogeographical patterns found in *Luciobarbus* seems to follow a close relation with the evolution of the Mediterranean Basin and the Alpine orogeny that led to the isolation of the Parathetys Lake. On the basis of mitochondrial and nuclear markers, we found three different clades composed by Iberian, one Greek species and north-African and Easter Asian species. The time-calibrated phylogenies and the biogeographical reconstruction led us to suggest different dispersion and vicariant events during Plio-Miocene, as well as a higher importance in the evolution and isolation of the Paratethys in the reconstruction of the historical biogeography of *Luciobarbus.*

1. INTRODUCTION

1.1 Cyprinids:

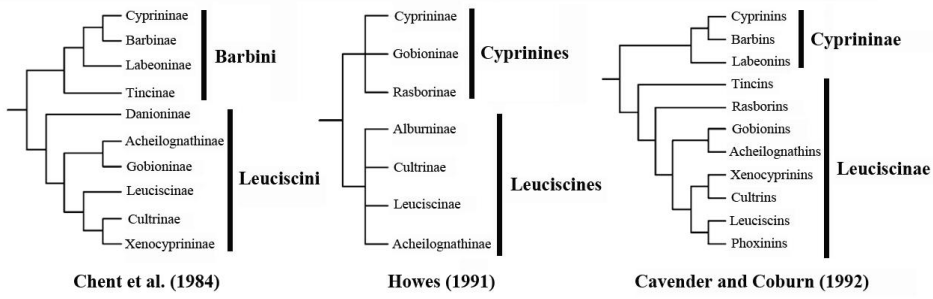
The Cypriniformes is one of the four orders belonging to the series Otophysi and to the superorder Ostariophysi, which is characterized by the presence of the Weberian apparatus, consider as a phylogenetically distinguishing character (Rosen & Greenwood, 1970). This structure enhance hearing and consist of a double chain of ossicles joining the air bladder to the inner ear, derived from the four first modified vertebrae immediately following the skull and connecting the swim bladder to the auditory system (Weber, 1820, Howes, 1984, Diogo, 2009). In the case of Cyprininae, the two first vertebrae of the Webber apparatus are fused.

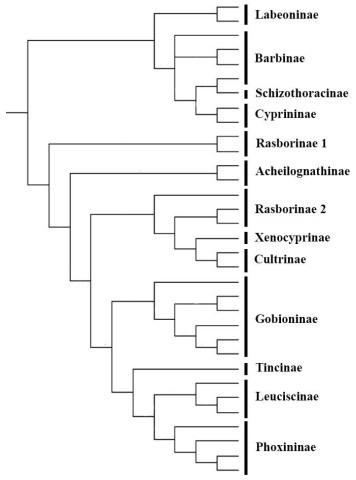
The Cypriniformes is the largest clade of freshwater fishes, counting with about 367 genera and about 3.000 species. Within Cypriniformes, the family Cyprinidae is the most species-rich family of vertebrates, comprised by freshwater fishes widely distributed in North America, Africa and Eurasia (Nelson *et al.*, 2016).



Phylogeny of major orders of the serie Otophysi (super order Ostraiophysi), extracted from Nelson *et al.* (2016)

The family Cyprinidae has been divided into different valid subfamilies throughout time by different authors, which have considered this family a complicated one from a taxonomic point of view. Since Chen *et al.* (1984), that recognized 10 subfamilies, to the 7 subfamilies proposed by Howes (1991) (Cyprininae, Gobioninae, Acheliognathinae, Leuciscinae, Cultrinae, Alburninae and Rasborinae). Later, Cavender and Coburn (1992), suggested the division of the family into two main lineages, Cyprininae and Leuciscinae; and Nelson (2016) recently recognized 12 subfamilies, between which the subfamilies Cyprininae and Barbinae can be found.





Phylogenies based on morphological characters adapted from Saitoh *et al.* (2006) and phylogeny proposed by Saitoh *et al.* (2006).

Nelson (2016) considers within the subfamily Cyprininae the following genera: *Aaptosyax, Anematichthys, Aulopyge, Barbus, Capoeta, Carassius* (Crucian Carp and Goldfish), *Catlocarpio, Cyclocheilichthys, Cyprinion, Cyprinus* (e.g., Common Carp, koi), *Gymnocypris, Luciobarbus, Luciocyprinus, Poropuntius, Rohtee,* and *Varicorhinus*.

For the subfamily Barbinae, he considers the following genera: *Acrossocheilus, Balantiocheilos, Barbus (barbels,barbs), Clypeobarbus, Diptychus, Haludaria, Hampala, Onchystoma, Oreichthys, Pseudobarbus, Puntius, Schizothorax, Sinocyclocheilus, Semiplotus, Spinibarbus* and *Tor.* This classification considers the genus *Luciobarbus* and *Barbus* as belonging to different subfamilies.

Nonetheless, the classification of the subfamily Cyprininae has been subjected to debate through time (Yu *et al.*, 2000, Nelson, 2006, Wang *et al.* 2007, Saitoh *et al.*, 2006, Chen *et al.* 2013, Yang *et al.* 2010, 2012a, 2012b, 2012c, 2015). The work of Yang *et al.* (2015), based on mitochondrial data, recovered the subfamily Cyprininae as monophyletic and proposed a subdivision into 11 tribes, from which three are new: *Probarbini* (new tribe), *Labeonini* Bleeker, 1859; *Torini* Karaman, 1971; *Smiliogastrini* Bleeker, 1863; *Poropuntinii*, *Cyprinini* Rafinesque 1815; *Acrossochelini* (new tribe), *Spinibarbini* (new tribe), *Schizothoracini* McClelland, 1842; *Schizopygopsini* Mirza, 1991; and *Barbini* Bleeker, 1859. In the same work, Yang *et al.* (2015) assigned the following genera to the tribe *Barbini* Bleeker, 1859: *Scaphidonticus* Vinciguerra, 1890; *Semiplotus* Bleeker, 1860; *Cyprinion* Heckel, 1843; *Aulopyge* Heckel, 1841; *Barbus* Cuvier & Cloquet, 1843; *Luciobarbus* Heckel, 1843; and *Capoeta* Valenciennes, 1842. The tribe *Barbini* is recovered as monophiletic through mitochondrial data and included *Cyprinion, Semiplotus,* and *Scaphiodonichthys* as members of *Barbini*, although its shows different nuclear lineages, since the paralogs were mixed in the nuclear tree.

1.2 Biogeographical models in the western Palearctic region

The Mediterranean region is considered among the richest biomes on Earth, with an extraordinary level of endemic species (Cowling *et al.*, 1996, Sauquet *et al.*, 2009). The freshwater fish fauna of the region is characterized by a relatively low number of fish families with most species belonging to the family Cyprinidae with a relatively high number of endemic species on the Mediterranean slope (Bănărescu & Coad, 1991, Kottelat & Freyhof, 2007). This local endemicity richness could be a consequence of the influence of different geological and climatic events through time, such as tectonic dynamics and climatic oscillations related to the glacial-interglacial periods and sea regressions (Bianco, 1998, Hrbek & Meyer, 2003). All these factors could have led to geographical isolation and continental connections, promoting vicariance and dispersal events in the populations, faunal relocation, river captures, confluence of alluvial plains and connections between catchments with subsequent isolation of populations (Tsigenopoulous *et al.*, 2002, Craw *et al.*, 2007, Marková *et al.*, 2010, Perea *et al.*, 2015).

It is commonly accepted that current geographical distribution of cyprinids is determined by a summary of historical changes related to the geological evolution of the region (Lundberg, 1993) since they are primary freshwater fishes with a low dispersal capacity and intolerance to salinity (Myers, 1938). During Cenozoic three main dispersal events have been invoked to explain the widely distribution of cyprinid fishes in Europe. These three events would have taken place during three different periods: Eocene-Oligocene, Middle Oligocene and Late Miocene.

El oldest dispersion event during Eocene-Oligocene is supported by fossil record. The oldest known fossil of the Cyprinidae is *Parabarbus sp.* from the Early Eocene (49.0 – 54.8 mya) in Obailinskaya formation in Kazakhstan (Sytchevskaya, 1986, 1989, Patterson C, 1993). While in Europe it is not until early Oligocene that fossils dated from 33-34 mya in

Czech Republic (Obrhelová, 1969, Cavender, 1991) shed light on the presence of the first cyprinids. Based on these findings the classical biogeographical explanation formulated first by Banarescu (1973) and that was called as the north dispersal theory (Doadrio & Carmona, 2003). This theory proposes that the current distribution of freshwater fishes in Europe resulted from a dispersion event through Eastern Asia into Siberia towards Central Europe and the Mediterranean countries (Banarescu, 1973, 1992). The North dispersal theory is not only supported by the fossil record but also by the historical distribution of other vertebrates and by the paleogeography of the region. Thus, the closure of the Turgai Strait at the end of Eocene had a huge effect on the distribution of biota and of many terrestrial organisms, without doubt (Rögl, 1997, 1999).

A posterior dispersion of cyprinid fishes have been proposed at the end of Oligocene, when fossil evidence of cyprinids such as leuciscine and "Alburnus" (Bohme, 2000, 1993, Obrhelová, 1990) would indicate a quickly diversification on the Balkanian-Anatolian-Iranian archipelago with a posterior colonization of Central Europe during the Alpine orogeny. This would have been possible once the Slovenian corridor was closed (around 19-20 mya) connecting the Balkanian-Anatolian landmasses to the rest of Europe in the Early-Middle Miocene (Perea *et al.*, 2010).

The most recent dispersal event could have occurred during the phase Lago Mare (Hsü *et al.*, 1977). This phase would have taken place during the Messinian Salinity Crisis, a period in which the Mediterranean lost connection with the Atlantic Ocean, although the basin would have not be completed dried and transformed in a network of freshwater lakes, allowing Circum-Mediterranean dispersal (Bianco, 1990). However, the formulation of these dispersal theories show a problem since they have not been supported by a suitable methodology and many of them have

been postulated in a narrative way, as an explanation of a phylogenetic work, in some cases.

Two hypothesis have been claimed for the processes of cyprinids colonization of southern peninsulas: One, postulated in which the colonization of the southern peninsulas would have been an old process that that took place since Oligocene to Pliocene, from Asia to Siberia and to Central Europe through river connections and freshwater lakes with a posterior colonization of the Iberian Peninsula before the Pyrenean-Alpine orogeny (Andeweg, 2002, Böhme, 2001; Popov *et al.*, 2004); and a second hypothesis, by Bianco, 1990, in which the colonization would be a more recent process, during the Messinian (around 5.3 mya), in which dispersal Nonetheless, posterior works did not found evidence to support this scenario since the origin and colonization of southern Peninsulas would have been much older than the MSC (Doadrio & Carmona, 2003, 2004, Levy *et al.*, 2009). This is supported by the presence of pre-Messinian Cyprinidae fossils such as *Rutilus* dated in the Tortonian, *Squalius* from the Middle Miocene (Gaudant, 1977, de la Peña, 1995).

1.3 The genus *Luciobarbus,* a brief summary on its evolution and biogeography

Initially, the genus *Luciobarbus* Heckel 1843 was considered a junior synonym of the genus *Barbus* until a morphological and osteological study of Mediterranean barbels suggested the monophyletic entity to a different *Barbus* sensu stricto and consider in a subgeneric category (Doadrio, 1990). Posterior morphological and molecular studies lead to its recognition at the generic level (Machordom & Doadrio, 2001a, Tsinegopoulous *et al.*, 2003, Kottelat & Freyhoff, 2007, Yang *et al.*, 2015). Within the genus *Luciobarbus* two lineages have been distinguished through time, one comprising species of *Luciobarbus* from the Iberian Peninsula and a second one comprised by the rest of the species within the

genus, in which North African species and Eastern species are included (Doadrio, 1990, Machordom *et al.*, 1995, 1998, Machordom and Doadrio, 2001a, 2001b, 2001c). The current systematic knowledge of the genus is based on several revisions counting with morphological, osteological and molecular approaches with over 35* species described so far (Geiger *et al.*, 2014, Casal-Lopez *et al.*, 2015, Doadrio *et al.*, 2016).

The genus *Luciobarbus* is tetraploid, shows high levels of local endemisms and is widely spread across freshwater systems of Asia, Greece, North Africa and southern Iberian Peninsula. All the species of the genus are primary freshwater fishes (non-salinity tolerant, Myers 1938) a characteristic that makes them a good model for biogeographical studies since they have limited dispersal to inland routes and its dispersion depends on the interconnectivity between freshwater systems through palaeogeographical changes which could lead to stream capture and piracity.

The first fossil of *Luciobarbus* in the Iberian Peninsula dates from late Miocene (around 6 mya) from the Guadix-Baza Basin (Doadrio & Casado, 1989). For the genus *Luciobarbus* several hypothesis have been suggested: A first one would be proposed by Bianco (1990) and supported by Durand *et al.*, (2002), and Tsinegopoulous *et al.*, (2003) by which the dispersal may have occurred during the brackish or freshwater phase (Lago Mare Phase, Hsu *et al.*, 1977, Penzo *et al.*, 1998) that followed the Messinian Salinity Crisis (around 5.5 mya), by which the Mediterranean would have not completely dried and would have received freshwater inflow from the Paratethys promoting a network of freshwater lakes that would have allowed Circum-Mediterranean dispersal. Nonetheless, some authors (Machordom & Doadrio, 2001) pointed out a series of unexplained facts, by which this hypothesis was questioned, such as the lack of extant *Luciobarbus* species from Greece to the Iberian Peninsula, the close phylogenetic relationship between south Iberian and Kabilian species and that no other cyprinid evidence of dispersion during Lago Mare phase.

There is presence of fossil remains before of Lago Phase Mare. A second hypothesis was proposed by Kosswig (1973) and reformulated by other authors (Doadrio, 1990, Machordom & Doadrio, 2001), by which *Luciobarbus* would have dispersed from the Middle East through North Africa (Doadrio, 1990, Doadrio *et al.*, 1998).

1.4. Biology and ecologic remarks of the genus *Luciobarbus*

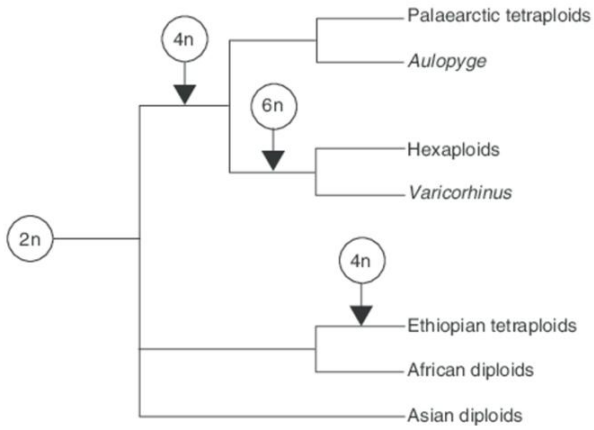
The genus *Luciobarbus* is characterized by medium to large fish species occurring in large rivers and downstream zones with slow-moving waters. During spawning period, they migrate upstream and the males of limnophylic species develop big nuptial tubercles at the end of the snout, an ornamentation not shown by reophilic species of North Africa, that develop nuptial tubercles all around the body. Females are usually bigger than males, and show longer anal fins, whereas male show longer pelvic fins than females. The species of *Luciobarbus* lay their eggs usually in the gravel bottom of the rivers and the female is usually accompanied by more than one male that will fertilize the eggs. The genus *Luciobarbus* comprises limnethic and rheophilic species with different habitat requirements showing a high degree of morphological and genetic differentiation. The body is elongated, thick anteriorly and laterally compressed posteriorly, with two pairs of barbels, thick and papillose lips. In most species the lower lip is thick and fleshy and in some the median part of the lip shows a lobe. They present frequently have denticulated the last single ray of the dorsal fin (Doadrio, 1990, Kottelat & Freyhoff, 2007, Banarescu and Bogutskaya, 2003).

1.5 Polyploidy in fishes

Polyploidy in fishes has been associated with traits including large body size, fast growth rate, long life and ecological adaptability (Uyeno & Smith, 1972; Schultz, 1980). Poliploidy has occurred independently in a variety of fish orders and that those most species-rich orders are known to include quite a large number of polyploid forms. Cypriniformes has shown

to be one of the orders with the highest number of polyploid species (Le Comber & Smith, 2004). Hybridization and introgression are common in freshwater fishes, in particular in the Cyprinidae, a highly speciose group,

and different levels of ploidy are observed (Lagier *et al.* 1977, Machordom & Doadrio, 2001, Alves *et al.*, 2001, Yang *et al.* 2005). Within the Cyprinidae, the genus *Barbus* sensu lato is characterized for being a species-rich group. For this group at least three polyploidization events have been suggested and tetraploidy appears to have evolved separately on two occasions from an ancestral diploid state, whereas the hexaploids appear to be monophyletic (Tsigenopoulos *et al.*, 2002). In this respect, other genera appear as sister groups of some monophyletic groups suggesting that the hexaploids may have originated via allopolyploidy (Guegan *et al.*, 1995, Machordom & Doadrio, 2001, Levin *et al.*, 2012). Thus, polyploidy, including allopolyploidy, does seem to have played a role in the speciation of this group (Machordom & Doadrio, 2001).



From Le Comber & Smith (2004), adapted from Machordom and Doadrio (2001)

Therefore, the use of nuclear markers in phylogenies has been particularly challenging through time. The information render by direct sequencing of nuclear markers results in a mix of gene copies that are not readable and the selection of the nuclear gene to be used for inferring phylogenetic relationships between groups is a key point to avoid misleading interpretations.

Within the genus *Barbus* and *Luciobarbus*, although the segregation of chromosomes during meiosis is disomic, all known species are tetraploid due to a second round on genome duplication (Collares-Pereira 1989, Collares-Pereira & Coelho, 1989, Collares-Pereira & Madeira, 1990). Due to the complete genome duplication of *Barbus*, most molecular studies have been based on mitochondrial DNA, allozyme electrophoresis and more rarely on microsatellites (e.g.,Machordom *et al.*1995; Chenuil *et al.* 1997, 1999; Zardoya & Doadrio 1999; Callejas & Ochando 2000, 2002; Doadrio *et al.*, 2002; Tsigenopoulos *et al.* 2002, 2003; Gante *et al.* 2011; Lajbner *et al.* 2009; Schreiber, 2009). Indeed, studies that include *Barbus* and employ nuclear sequence data use at best one of the paralogous loci (Mayden *et al.* 2009; Marková *et al.* 2010). Some authors have approached this issue by cloning individual amplicons to identify paralogs (Bart *et al.*2010; Saitoh *et al.* 2006), in order to separate gene copies and designed sets of paralog-specific primers (Gante *et al.*, 2011, Yang *et al.*, 2015).

1.6 Objectives

The general objective of this dissertation was to analyse the patterns and processes of evolution and diversification of the genus *Luciobarbus* in the western Paleartic through the use of molecular markers, biogeographical methodologies and taxonomic remarks in order to study its phylogenetic relationships and biogeographic patterns. For this, several points were proposed as specific objectives:

1. Contribute to the systematics and taxonomy of the group by the description of new taxa within the genus that remained undescribed.

2. Suggest a phylogenetic hypothesis for the genus *Luciobarbus* by using mitochondrial and nuclear markers.

3. Suggest a biogeographical hypothesis for patterns and modes of colonization of the genus *Luciobarbus* in the western Paleartic.

4. Understand phylogeographical patterns of an endemic species of the southern Iberian Peninsula: *Luciobarbus sclateri*

5. Compared population structures and patterns of diversification of allopatric populations of *Luciobarbus* and test whether the Messinian connections between southern Iberia and Northern Africa played a key role on the diversification of the lineages of *Luciobarbus* on both sides of the Strait

6. Review the phylogeny of the genus by the information render by mitochondrial and nuclear markers

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2. CHAPTER I

This chapter is based upon the manuscript:

Casal-López, Perea, S.,Yahyaoui, A. & Doadrio, I., 2015 Taxonomic review of the genus *Luciobarbus* Heckel, 1843 (Actinopterygii, Cyprinidae) from northwestern Morocco with the description of three new species. Graellsia 71(2):27

TAXONOMIC REVIEW OF THE GENUS LUCIOBARBUS HECKEL, 1843 (ACTINOPTERYGII, CYPRINIDAE) FROM NORTHWESTERN MOROCCO WITH THE DESCRIPTION OF THREE NEW SPECIES

ABSTRACT

The genus *Luciobarbus* in Morocco presents high diversification, in contrast to the generally impoverished freshwater fish fauna from North Africa. Within Morocco the northern area is one of the least studied territories, due to both its historical background and the limited accessibility of many regions. Previous phylogenetic studies identified *Luciobarbus* populations that are morphological and genetically differentiated, to the same extent as others, already recognized as separate species. The aim of this work was to describe those populations as distinct species, based on morphological, meristic, and genetic traits.

Introduction

The genus *Barbus sensu lato* includes more than 800 species and it is one of the genera with highest number of species among vertebrates. It is a polyphyletic assemblage with different genetic and morphologic features. In fact, in recent years, *Barbus* phylogenetic studies have led to its division into different genera (Karaman, 1971; Machordom & Doadrio, 2001a; Yang *et al.*, 2015).

European and North African species previously included in genus the *Barbus* Cuvier & Cloquet, 1843 are presently included in genera (Kottelat & Freyhof, 2007) *Barbus* and *Luciobarbus* Heckel, 1843. The genus *Luciobarbus* was initially assigned to the Asian species *Luciobarbus esocinus* Heckel, 1843 but was long considered as a synonym of *Barbus* Cuvier &

Cloquet, 1843. Notwithstanding, a phylogenetic study using morphological characters revealed
that most limnophilic circum-Mediterranean species occurring in northern Africa, the Mediterranean peninsulas, and Near East were monophyletic (Doadrio, 1990). This monophyletic clade was included within the subgenus *Luciobarbus* (Doadrio, 1990), which with the advent of molecular studies, was better defined and recognized at the generic level (Machordom & Doadrio, 2001b; Tsigenopoulos *et al.*, 2003; Griffiths *et al.*, 2004; Kottelat & Freyhof, 2007; Yang *et al.*, 2015).

Molecular studies of the genus *Luciobarbus* have, so far, been based on isoenzyme analysis (Machordom *et al.*, 1995, 1998; Doadrio *et al.*, 1998) and sequencing of mitochondrial genes (Zardoya & Doadrio, 1999; Zardoya *et al.*, 1999; Machordom & Doadrio 2001b; Doadrio *et al.*, 2002; Tsigenopoulos *et al.*, 2003). The sequencing of nuclear genes has been constrained by the tetraploid condition of the species of this genus (Yang *et al.*, 2015).

Results of previous molecular studies have revealed a strong *Luciobarbus* population structure in North Africa (Berrebi *et al.*, 1995; Doadrio *et al.*, 1998; Machordom *et al.*, 1998; Machordom & Doadrio, 2001b). Sixteen populations, genetically isolated during the late Miocene and Pliocene, were recognized in North Africa, some of them assigned to different species (Machordom & Doadrio, 2001b). Within North African populations, those of northwestern Morocco, inhabiting areas ranging from the basins of the Laou River (on the Mediterranean slope in the north) to the Kasab River (on the Atlantic slope in the southwest), have been clustered together by genetic studies (Machordom & Doadrio, 2001b; Geiger *et al.*, 2014). However, there are few taxonomic studies focusing on *Luciobarbus* populations of northwestern Morocco, due to its traditional isolation and limited accessibility (Almaça, 1966, 1968, 1970; Doadrio, 1990). For these reasons northern Morocco was ignored, when taxonomic African

ichthyological studies were conducted by the French Geographical Society, in the early twentieth century.

Some *Luciobarbus* populations, mainly from Laou, Grou and Sebou Basins, belonging to northwestern Morocco present high genetic differentiation in several molecular markers, such as isozymes and the mitochondrial cytochrome *b* gene, compared to other populations of the genus and constitute a monophyletic group (Machordom *et al.*, 1998; Machordom & Doadrio, 2001b; Geiger *et al.*, 2014). Nonetheless, only morphological works have been carried out for the Sebou Basin (Almaça, 1966, 1968, 1970; Doadrio, 1990).

The aim of our study was to extend molecular and morphological works to other populations from northwestern Morocco. Thus, our goal was to clarify, with a more complete sampling, the taxonomy of *Luciobarbus* in northwestern Morocco with an integrative approach and unravel whether the diversity found corresponds to new taxa that may not have been formally described so far. In this case, we will formally describe these new taxa.

Material and Methods

MORPHOLOGICAL ANALYSES

The taxonomy of the population of northwestern Morocco was based on 47 specimens from Laou Basin, 43 specimens from Loukos Basin, 22 specimens from Hachef Basin, 55 specimens from Sebou Basin, 33 specimens from Bou Regreg Basin and 23 specimens from Kasab Basin (Fig. 1, Table 1).

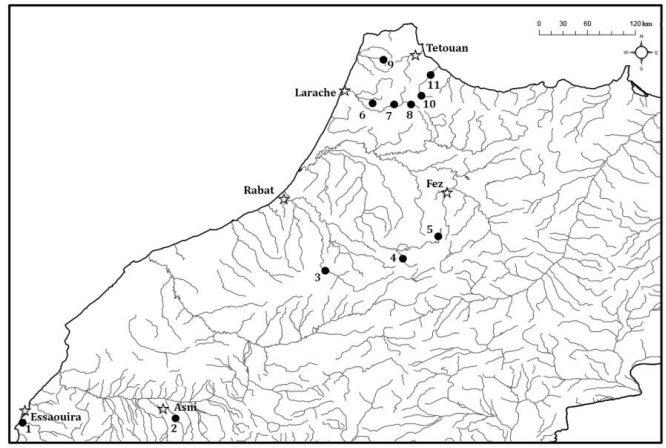

Fig. 1. Geographic distribution of *Luciobarbus spp*. and sampling localities. The numbers on map correspond to localities in Table 1.

The material studied consisted of the following locations and number of specimens: 25 adult specimens from the Laou River, Laou Basin, Derdara, (35.118986, -5.288900), Morocco (Voucher numbers: MNCN 290.639-290.652, 290.655, 290.657-663, 290.665-667); 22 specimens from the Laou River, Laou Basin, Beni Ferten (35.353254, -5.184840), Morocco (Voucher numbers: MNCN 284.939-940, 284.942-945, 284.947-284.948, 284.950-951, 284.953-964); 43 specimens from the Loukos River, Loukos Basin, Souk Had, Laghdir (35.02624,-5.404660), Morocco (Voucher numbers: MNCN: 280.162-163, 280.165, 280.168, 280.170, 280.172-174, 280.176-181, 280.183, 280.185-186, 290.671-696); 22 specimens from the Hachef River, Hachef Basin, Dar Chaoui (35.526763,-5.713771), Morocco (MNCN 290.707-714; 290.716-722; MNCN 290.725-731); 32 specimens from the Ifrane River, Sebou Basin, Ouad Ifrane, (33.296957, -5.492639) Morocco (voucher numbers: MNCN 279.711-729, 290.731, 279.733-744); 23 specimens from the Tizguit River, Sebou Basin, Ifrane (33.549241, -5.097144), Morocco (Voucher number: MNCN 71675-697); and 33 specimens from the Grou River, Bou Regreg Basin, Sebt Ait Rahhou (33.164678, -6.366963), Morocco (Voucher numbers: MNCN 71725-746, 71918-28). For comparative

purposes, we analyzed 23 specimens of *Luciobarbus ksibi* Boulenger 1905 from species type locality in the Kasab River, Kasab Basin, Essaouira (31.465857,-9.759850) Morocco (Voucher numbers: MNCN 105.460-65, 105.469, 234.925-29, 280.483-485, 71220-21, 71223-26, 71230, 290.670). All sampling sites (Fig. 1) presented similar riverine morphology, with clear water and fast current and gravel bottom, with the exception of the Kasab River, in Essaouira, which showed a more marked seasonal regime and poorer water conditions.

Table 1. Sampling localities for *Luciobarbus spp*. Code is the number on the phylogenetic tree. G= Genetic, M= Morphometry.

Species	River	Locality	Basin	Analyses	Code	GenBank	Map
L. ksibi	Kasab	Essaouira	Kasab	G,M	K6-K10	KT003951-55	1
L. ksibi	Reraia	Asni	Tensift	G	K1-K5	KT003956-60	2
L. sp1	Tizguit	Ifrane	Sebou	M			4
L. sp1	Ifrane	Ouad Ifrane	Sebou	G,M	S1-S6	KT003941-45	5
L. sp2	Tattofte	Ouled Soltane	Loukos	M			6
L. sp2	Loukos	Mouries	Loukos	M			7
L. sp2	Loukos	Souk Had, Laghdir	Loukos	G	RK1-RK5	KT003936-40	8
L. sp2	Hachef	Dar Chaoui	Hachef	G,M	RH1-RH5	KT003931-35	9
L. sp2	Laou	Derdara	Laou	G,M	RL1-RL5	KT003926-30	10
L. sp2	Laou	Beni Ferten	Laou	M			11
L.sp3	Bou Regreg	Sebt Ait Rahhou	Grou	G	B1-B5	KT003946-50	3
L. capito	Terek	Kizlyar	Terek (Russia)	G	*L. capito*	AF045975	

Twenty-three morphometric measurements (in mm) and nine meristic variables were recorded from digital photographs using TpsDig v.1.4 (Rohlf, 2003). The following abbreviations were used for morphometric and meristic characters: A, anal fin rays; AFH, anal fin height; AFL, anal fin length; APL, anal peduncle length; BL1, first barbel length; BL2, second barbel length; BD, body depth; LBD, lowest body depth; C, central caudal fin rays; CFL, caudal fin length; CPL, caudal peduncle length; D, dorsal fin rays, DFL dorsal fin length; DFH dorsal fin height; ED, eye diameter; HL, head length; LL lateral line scales; P, pectoral fin rays; PFL, pectoral fin length; PrAD, pre-anal distance; PrDD, pre-dorsal distance; PrOL, pre-orbital length; PrPD,

pre-pectoral distance; PrVD, pre-ventral distance; PsOL, postorbital length; PVL, pectoral-ventral length; RSA, scale rows above lateral line; RSB scale rows below lateral line; SL, standard length; V, ventral fin rays; VFL, ventral fin length; VE, Number of vertebrae. The number of vertebrae was obtained by direct counting on X-ray images of individuals from all populations sampled.

After constructing the measurement matrix, Burnaby's method was used to correct size effect (Burnaby, 1966; Rohlf & Bookstein, 1987). All analyses were conducted with the corrected matrix. Morphometric and meristic characters were analyzed independently. A two-way analysis of variance (ANOVA) comparing morphometric characters was conducted to test for sexual dimorphism and variation among populations. To identify the variables that contributed most to the variation between populations, a principal components analysis (PCA) was performed using the covariance matrix for morphometric characters.

MOLECULAR ANALYSES

For the molecular approach, we analyzed samples corresponding to individuals of *Luciobarbus spp.* from Sebou, Laou, Hachef, Loukos and Bou Regreg Basins; and *Luciobarbus ksibi* from the Kasab Basin (Table 1). The species *Luciobarbus capito* (Güldenstädt, 1773) was selected as outgroup based on previous phylogenetic analyses (Zardoya & Doadrio, 1999). Total genomic DNA was extracted from fin-clip tissue using the commercial kit Biosprint15 for tissue and blood (Qiagen). For each specimen, the complete region (1140bp) of the mitochondrial cytochrome *b* (cyt*b*) was amplified. Primers and protocols used for PCR for cyt*b* followed Machordom & Doadrio (2001b). After checking PCR products on 1% agarose gels, they were purified by ExoSAP-IT ™ (USB) and directly sequenced on MACROGEN service using a 3730XL DNA sequencer. All sequences were deposited in the GenBank database (Accession Numbers: KT003926-KT003960).

PHYLOGENETIC ANALYSES

Phylogenetic analyses were performed using Bayesian inference (BI) implemented in MrBayes v.3.2 (Ronquist *et al.*, 2012). The Akaike Information Criterion (Akaike, 1973) implemented in jModeltest (Posada, 2008) was used to determine the evolutionary model that best fit the data. In this case TrN+I model was selected (R(a) [AC] = 1.0000, R(b) [AG] = 92.1342, R(c) [AT] = 1.0000, R(d) [CG] = 1.0000, R(e) [CT] = 20.3361, R(f) [GT] = 1.0000, p-inv = 0.7900). BI was performed using two independent runs of four Markov Montecarlo coupled chains (MCMC) of 10^6 generations each, to estimate the posterior probability distribution. Topologies were sampled every 100 generations, and majority-rule consensus tree was estimated after discarding the first 10% of generations. Robustness of clades was assessed using Bayesian posterior probabilities. The average genetic distances among *Luciobarbus* populations were calculated for each gene using MEGA package v.6.0 (Tamura *et al.*, 2013) according to the uncorrected-*p* distances.

Results and Discussion

COMPARISON OF MORPHOLOGY AMONG POPULATIONS

Two-way analysis of variance (ANOVA), testing for sexual dimorphism and differentiation among populations, showed significant differences ($p<0.05$) for the variables standard length, postorbital length, and anal fin size (Table 2). In absolute values all variables were greater in females, but proportionally to the standard length postorbital length was longer in males (Table 3). A non-biological interpretation could explain the differences in postorbital length by sex, therefore we performed a test of Mann-Whitney-Wilcoxon at $p < 0.05$, with postorbital length and sex as variables, for each population independently.

The null hypothesis for identical postorbital length between males and

females for each population was not rejected in all populations (Sebou Basin *z*=-0.810; Bou Regreg Basin *z*=-0.868; Kasab Basin *z*=-1.692; Loukos Basin *z*=-0.480; Laou Basin *z*=-0.343; Hachef Basin *z*=-0.032). Therefore, the differences in postorbital length were not explained by sex and were an effect of population differences.

Table 2. Two-way analysis of variance (ANOVA) for sexual dimorphism, population variation, and their interaction. Significant differences p<0.05 (*); p<0.01 (**). N=163 males and n=60 females. Acronyms are defined in the Material and Methods.

Variables	Sexual dimorphism (f/p-value)	Population Variation (f/p-value)	Sex/pop variation (f/p-value)
SL	4.51/*	53.38/**	2.37/
HL	3.06/	26.74/**	1.94/
PrOL	1.45/	13.36/**	2.08/
ED	0.22/	11.32/**	0.57/
PsOL	22.32/**	469.5/**	7.58/**
B1L	3.45/	10.09/**	2.17/
B2L	1/	21.07/**	0.19/
PrDD	1.66/	78.49/**	2.14/
PrPD	0.01/	44.54/**	4/**
PrVD	3.18/	68.28/**	3.55/*
PrAD	1.75/	56.71/**	3.5/*
CPL	0.96/	11.14/**	1.92/
APL	0.51/	3.68/*	4.39/**
PVL	0.01/	12.71/**	0.51/
BD	3.13/	9.64/**	1.34/
BLD	0.001/	6.93/**	1.61/
DFL	2.04/	2.9/	0.137/
DFH	3.31/	27.42/**	2.32/
PFL	0.10/	23.08/*	1.8/
VFL	2.42/	14.26 /**	0.19/
AFL	4.26/*	16.61/	2.37/
AFH	11.46/**	29.88/**	2.06/
CFL	0.87/	16.08/**	1.41 /

Females had proportionally higher and longer anal fins (Table 3). Sex differences in the size of anal fins have been found in other *Luciobarbus* species, and are probably associated with the female's use of the anal fin to excavate nests in the riverbed (Banarescu & Bogutskaya, 2003). Nuptial tubercles were present on the snout and head of males, particularly around the preorbital region.

Table 3.— Morphometric variables showing significant sexual dimorphism ($p<0.05$). Values are mean (minimum-maximum). Acronyms are defined in the Material and Methods.

Variables (mm)	Males n=163	Females n=60
SL	106.2 (53.1-232.8-75)	157.7 (82.1-240.1)
PsOL	13.5 (6.6-30.8)	18.9 (9.6-29.6)
AFL	8.3 (3.74-17.8)	12.3 (6.2-19.3)
AFH	18.3 (10-41.6)	27.5 (15-44.8)

To deal with the presence of sexual dimorphism in anal fin size, we removed AFL and AFH from posterior analyses. Most morphometric variables showed significant differences between populations in the two-way ANOVA analysis (Table 2). An analysis of body proportions based on Kruskal-Wallis and Mann-Whitney *post hoc* comparisons, was used to detect differences in body shape that can be masked if only linear untransformed measurements are taken into account (Table 4). We grouped the populations from the Rif Mountains (Laou, Loukos, and Hachef Basins) based on mitochondrial DNA analysis and previous genetic studies (Machordom *et al.*, 1998, Machordom & Doadrio, 2001b; Geiger *et al.*, 2014). The populations from Sebou Basin showed a shorter predorsal distance and a longer caudal peduncle than the other *Luciobarbus* populations studied, due to the more anterior position of the dorsal fin (Fig. 2, Table 5).

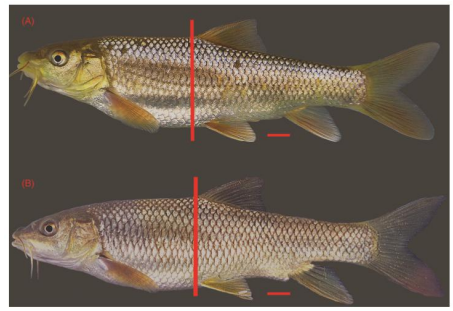

Fig. 2. Comparison of two females of similar standard length from (A) Sebou Basin and (B) Laou Basin (Rifian population) showing differing position of the dorsal fin with respect to ventral fin insertion.

The Sebou population exhibited greater postorbital length with respect to the other studied populations, and therefore the ratio SL/PsOL in the Sebou population was the lowest (Table 5). The caudal peduncle was higher in *Luciobarbus ksibi* (Kasab population) than in the other studied populations, and the ratios SL/CPL and SL/APL, were lowest. Barbels were longest in *Luciobarbus ksibi*. Preorbital length was significantly shorter in the Bou Regreg population compared to the other populations studied.

Lateral line scales were more numerous in the Bou Regreg population than in other populations. Scales numbers on the superior transverse line were higher in Bou Regreg and Rifian populations than in *L. ksibi* and Sebou populations, while the number of scales on the inferior transverse line was lower in Rifian populations and *L. ksibi* (Table 5).

Table 4. Ratios of morphometric variables and scale count. Values are mean (minimum and maximum). Acronyms are defined in the Material and Methods.

Measurements	**Rif populations (n=112)**	**Sebou (n=55)**	**Bou Regreg (n=33)**	***Luciobarbus ksibi* (n=22)**
Sl/PrDD	1.87(1.79-1.95)	1.93 (1.79-2.03)	1.85 (1.78-1.96)	1.85 (1.73-1.98)
SL/CPL	2.81 (2.56-3.06)	2.7 (2.54-2.91)	2.79 (2.63-2.91)	2.82 (2.63-3.17)
HL/ PsOL	2.21 (1.65-2.50)	1.61 (1.29-1.81)	2.04 (2.47-3.41)	2.23 (2.03-2.57)
HL/PrOL	2.74 (2.35-3.23)	2.82 (2.48-3.49)	2.97 (2.47-3.5)	2.78 (2.4-3.1)
SL/BLD	9.11 (7.48-10.92)	8.72 (7.57-10)	8.38 (8.06-8.7)	7.8 (7.36-8.27)
CPL/BLD	3.24 (2.46-3.83)	3.24 (2.67-3.75)	3 (2.81-3.26)	2.78 (2.6-2.99)
APL/BLD	1.64 (1.25-2.07)	1.58 (1.37-1.83)	1.55(1.39-1.67)	1.5 (1.29-1.87)

HL/L1B	4.16 (2.78-6.87)	3.96 (3.03-5.27)	4.11 (3.8-4.5)	3.5 (2.93-4.05)
HL/L2B	3.27 (2.48-5)	3.1 (2.4-4.74)	3.29 (3.05-3.52)	2.65 (2.26-3.25)
LL	43.9 (46-42)	43.7 (41-47)	46.5 (45-50)	43.7 (41-47)
RSA	8.8 (8.5-9.5)	7.5 (7.5-8.5)	9.6 (8.5-10.5)	8.3 (6.5-8.5)
RSB	4.7 (3.5-5.5)	5.5 (4.5-6.5)	6.74 (6.5-7.5)	4.98 (4.5-5.5)

The principal component analysis divided the populations into three groups corresponding to *Luciobarbus ksibi* and populations of the Rifian and the Sebou Basins. The population of the Bou Regreg Basin was included in the morphometric variability of Rifian populations (Fig. 3). Nevertheless, the Bou Regreg population could be discriminated from the other studied populations by the number of scales on the body (Fig. 4).

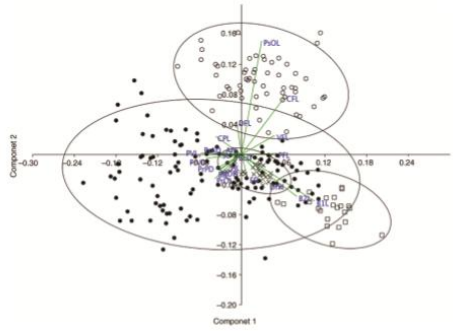

Fig. 3. Variables that most contributed to the PCA analysis. Dots, Rifian populations. Squares, Luciobarbus ksibi from Kasab Basin. X, Population from Bou Regreg Basin. Circles, population from Sebou Basin. Abbreviations are defined in Materials and Methods.

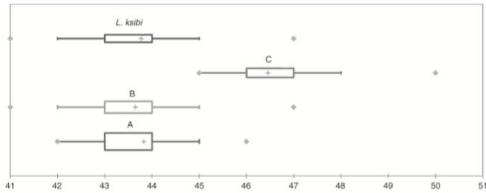

Fig. 4. Box-plots of lateral line scale numbers. A, Rifian population; B, Sebou Basin population; C, Bou-Regreg Basin population.

The eigenvalues of the two first principal components, with the Burnaby-corrected matrix, explained most of the variance (Table 6). The highest values for eigenvectors in both males and females, and,

consequently, the variables that contributed most to the ordination in the PCA were: postorbital and preorbital length, caudal and dorsal fin size, and barbel length (Table 6).

Table 5. Eigenvalues and eigenvector for the principal components (PC1-PC3) of 21 morphometric variables for all Luciobarbus populations. Acronyms are defined in the Material and Methods. In bold, variables with the highest eigenvectors for each PC.

Variables	PCI	PCII	PCIII
Eigenvalue	0.0073	0.0044	0.0029
% variance	29.1	17.56	11.48
	Eigenvectors		
SL	-0.1416	-0.0053	0.0137
PrDD	-0.1419	-0.0904	0.0254
PrPD	-0.1741	-0.0915	-0.0420
PrVD	-0.2401	-0.0236	-0.0667
PrAD	-0.1961	0.0014	-0.0377
PVL	-0.2772	0.0095	-0.0804
CPL	-0.1750	0.1154	0.0243
APL	-0.1185	0.0260	0.1110
BD	-0.1550	-0.1024	-0.0021
BLD	-0.0296	-0.0078	-0.0763
HL	-0.0841	-0.0731	-0.0450
PrOL	-0.1817	-0.1536	-0.0761
ED	0.0442	-0.1513	-0.1591
PsOL	0.1385	**0.7205**	-0.3674
B1L	**0.5001**	-0.2952	**-0.4918**
B2L	**0.3794**	-0.2658	-0.1933
PFL	0.2412	0.0068	0.2772
VFL	0.2282	0.1219	0.1780
DFL	-0.0322	-0.2115	-0.0283
DFH	0.1795	0.3645	**0.4958**
CFL	0.2966	**0.2586**	**-0.4046**

Osteological Features

The hardness of the last single ray of the dorsal fin (DFR) and its number of denticulations frequently has been used in the taxonomy of *Luciobarbus* (Almaça, 1970). As Doadrio (1990) pointed out, there is ontogenetic variability in DFR, and comparison of the characteristics of DFR among species, for taxonomic purposes, must be done using adult specimens. We found *L. ksibi* presents strong denticulations but only over 2/3 of the DFR length. Denticulations of the DFR in specimens of the Bou Regreg population were deep and the length of these denticulations was greater than the width of the ray. Dorsal fin rays of the Rifian and Sebou Basins populations were similar, slightly stronger in Rifian populations than in the Sebou population.

The skulls of *L. ksibi* and Sebou populations are shorter and wider than that of the Bou Regreg and Rifian populations. The ethmoid bone in the Sebou population is narrower than in other studied populations, especially pronounced compared to the Rifian population (Fig. 1S). In the skull lateral view, the opercular bone appeared longer in the Sebou population than in other populations (Fig. 2S). Infraorbital bones are larger in *L. ksibi* than in other populations, and the second infraorbital bone is longer in Rifian than in other populations and usually exhibits four pores (Fig. 3S). The pharyngeal bone is thinner in the Bou Regreg populations and wider in *L. ksibi*. The dorsal branch of the pharyngeal bone forms a closed angle with respect to the inferior branch in Rifian population and *L. ksibi* (Fig. 4S).

Molecular Data

Phylogenetic analyses based on the cyt*b* gene supported four main clades in the tree, corresponding to the populations of the Rif, Sebou, and Bou Regreg Basins and *Luciobarbus ksibi* (Fig. 5). The genetic distances found among populations are presented in Table 7. Genetic distances within Sebou, Bou Regreg, Rifian populations and *L. ksibi* ranged between 0-0.6% but between these populations were 1.7-3.9%. The minimum value was found between *L. ksibi* and Bou Regreg populations (Table 7).

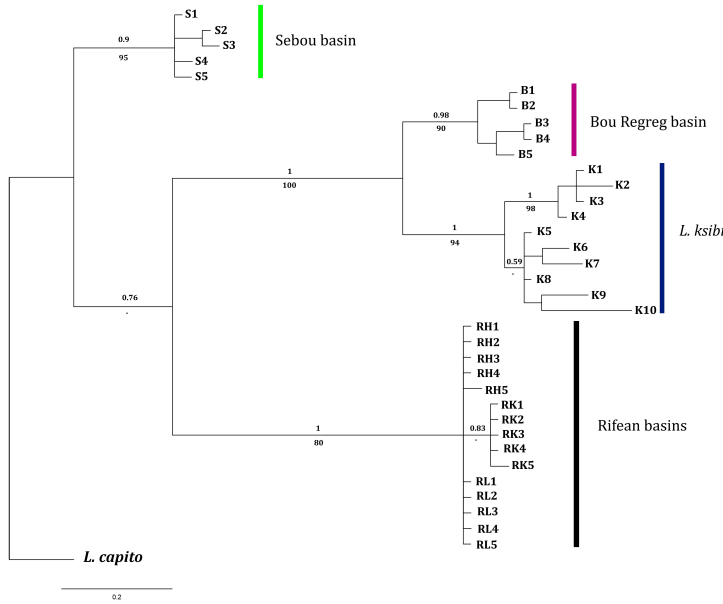

Fig. 5. Phylogenetic tree rendered by Bayesian Inference of the cytochrome b gene. Numbers on branches indicate posterior probability values. Abbreviations of localities are defined in Table 1.

Table 7.— Genetic distances for complete mitochondrial cytb gene. Uncorrected genetic distances between populations are presented below diagonal. In the diagonal uncorrected genetic distances within populations are shown.

	Laou (Rif)	**Hachef (Rif)**	**Loukos (Rif)**	**Sebou**	**Bou Regreg**
Hachef	0.0				
Loukos	0.2	0.2			
Sebou	3.2	3.2	3.2		
Bou Regreg	3.9	3.9	3.9	3.1	
L. ksibi	3.8	3.8	3.9	3.7	1.7

The distances are of similar range as those between well recognized species of cyprinid fish (Doadrio *et al.*, 2002, 2007a, 2007b; Doadrio & Carmona, 2003, 2006; Doadrio & Madeira, 2004; Robalo *et al.,* 2005; Doadrio and Elvira, 2007; Domínguez-Domínguez *et al.*, 2007, 2009).

These results confirm the differences found with allozyme analyses (Machordom *et al.*, 1998). Allozyme studies found 23 polymorphic loci, four of which (MPI, GPI-5, IDHP-2, CK) were diagnostic, with a probability criterion of 99% of correct assignment to Rifian populations with respect to the Sebou Basin. Between the Sebou Basin (Ifrane population) and Grou Basin three diagnostic loci were found (GPI-2*, GPI-5* and PGDH-1). The population of the Grou River (Bou Regreg Basin) exhibited a unique diagnostic locus (*GPI-2* 118*) with respect to all *Luciobarbus* populations (Machordom *et al.,* 1998). Populations from Sebou, Rif and Bou Regreg basins were identified as different taxa by other authors based on the molecular differences found, but were not formally described as new so far (Machordom *et al.,* 1998).

TAXONOMY OF *LUCIOBARBUS LABIOSUS* (PELLEGRIN, 1920)

Initially the population from Sebou Basin in Morocco was considered to belong to *Barbus setivimensis* Valenciennes, 1842, which was originally described for populations of the Setif Basin in Algiers (Pellegrin, 1920). Within the Sebou population of *B. setivimensis*, Pellegrin (1920) found specimens with well-developed lips that this author described as a variety, *B. setivimensis var. labiosa* Pellegrin, 1920. This development of the lips in some specimens of the former genus *Barbus sensu lato*, currently within *Luciobarbus,* is common, and Pellegrin (1922) described another variety as *Barbus massaensis var. labiosa* Pellegrin, 1922. As Almaça (1968, 1970) stated, Pellegrin (1920) did not describe the var. *labiosa* as a subspecific category or geographic form because he found typical specimens of *B. setivimensis* and some specimens with more developed lips in the same locality. For this reason Article 45.6.4 of the International Code of Zoological Nomenclature was not applicable:

> *"45.6.4. it is subspecific if first published before 1961 and its author expressly used one of the terms "variety" or "form" (including use of the terms "var.", "forma", "v." and "f."), unless its author also expressly gave it infrasubspecific rank, or the content of the work unambiguously reveals that the name was proposed for an infrasubspecific entity, in which case it is infrasubspecific"*

Subsequent molecular studies showed that populations of *L. setivimensis* of the Setif Basin in Algeria and those of the Sebou Basin in Morocco were not monophyletic. Thus, mitochondrial genes of species of *Luciobarbus* showed that *L. setivimensis* from the Setif Basin is a sister group of the Iberian species, while populations of *L. setivimensis* from the Sebou Basin were closer to North African species (Machordom & Doadrio, 2001b). For this reason, and in the absence of another valid name for the populations of the Sebou Basin, molecular studies have referred to Sebou populations as *Luciobarbus labiosa* (Machordom & Doadrio, 1993, 2001b; Doadrio, 1994; Geiger *et al* 2014).

Hence, populations from the Sebou Basin do not have a valid specific name at the present time, and we describe here this population as a new species.

DESCRIPTION OF *LUCIOBARBUS* POPULATIONS

The high degree of morphological and genetic differentiation of *Luciobarbus* populations endemic to the region of the Strait of Gibraltar in North Africa justifies the consideration of these populations as distinct species. No available name for these populations exists, and therefore three new species are described in this study.

Fig. 6. Holotype of Luciobarbus maghrebensis from the Ifrane River, Sebou Basin, Ouad Ifrane, Morocco. MNCN 279.718.

Luciobarbus maghrebensis Doadrio, Perea & Yahyaoui, **sp. nov.** urn:lsid:zoobank.org:act:3FE3F718-F050-44F5-93DC-C5ABFE3C8674

TYPE MATERIAL: Holotype: Fig. 6, Table 8. MNCN 279.718 female, 135.6 mm (SL); Ifrane River, Sebou Basin, Ouad Ifrane, Atlantic slope in Morocco (33.296957, -5.492639) (Fig. 1); 27/3/2013. Collected by (Coll.) Doadrio, I; Yahyaoui, A; González, E. G.; Perea, S.

Paratypes: Table 8. MNCN 279.711-717, 279.719-29, 279.731, 279.733-744: Thirty-one specimens from the Ifrane River, Sebou Basin, Ouad Ifrane, Atlantic slope in Morocco (33.296957,-5.492639); 27/3/2013. Coll. Doadrio, I; Yahyaoui, A; González, E. G.; Perea, S. MNCN 71675-697: Twenty-three specimens from Tizguit River, Sebou Basin, Ifrane, Atlantic slope in Morocco (33.549241,-5.097144). 19/4/1991. Doadrio, I; Garzón, P.

Holotype and a series of paratypes (55 specimens) have been deposited at the Fish Collection of the Museo Nacional de Ciencias Naturales, (Madrid, Spain).

Table 8.— Morphometric and meristic measurement of the holotype and paratypes of *Luciobarbus maghrebensis*

Morphometric measurement	Holotype MNCN 279718	Paratypes n= 54		
	Measurements (mm)	Range	Mean	Standard Deviation
SL	135.6	70-170.6	109.9	25.2
PrDD	68.8	33.4-85.7	56.8	12.2
PrPD	36.2	18.6-43.6	28.2	6.4
PrVD	75.6	40.2-93.2	59.7	14.3
PrAD	104.9	54.7-130.7	84.1	22.3
PVL	39.9	19.2-52	32.6	8.4
CPL	51.3	24.2-67.6	41.4	10.6
APL	25.2	12.1-32.9	20.5	5.0
BD	34.7	18.7-43.6	28.0	6.4
BLD	16.1	7.7-18.5	12.3	2.6
HL	33.7	17.1-38.8	26.6	5.3
PrOL	12.2	6.0-14.4.1	9.4	2.1
ED	7.7	4.2-8.2	5.7	1.1
PsOL	14.4	6.8-19.1	13.7	2.4
B1L	10.0	3.6-11.2	7.0	1.8
B2L	14.4	4.6-15.6	9.2	2.6
PFL	24.4	10.7-30.9	20.2	4.3
VFL	21.5	11.5-26	17.6	3.5
DFL	17.8	9.1-3.1	15.0	3.1
DFH	24.8	11.2-30.6	18.6	4.8
AFL	8.7	5.1-26.8	8.6	3.0
AFH	24.4	10.9-31	18.6	4.2
CFL	32.6	17.3-40.1	25.7	5.3
LL	44	41-47	43.7	1.2
RSA	7.5	7.5-8.5	7.6	0.2
RSB	5.5	4.5-6.5	5.5	0.4

SUPPLEMENTARY MATERIAL: Lectotype of *Barbus setivimensis* var. *labiosa* Pellegrin, 1920: MNHN-IC-1920-0215, Fez, Morocco. Coll. M. CH. Allaud. Paralectotypes of *Barbus setivimensis* var. *labiosa* Pellegrin, 1920: MNHN-IC-1920-0212 and MNHN-IC-1920-0214, Fez, Morocco.

DIAGNOSIS: Differs from other known species of *Luciobarbus* by the following combination of characters: 41-47 scales on the lateral line ($\bar{x}$ =43.7 Median=44); 7.5-8.5 ($\bar{x}$ =7.5 Median=7.5) above lateral line and 4.5-6.5 ($\bar{x}$ =5.47, Median=5.5) below lateral line. Insertion of the ventral fin is posterior to dorsal fin origin. The last single fin ray is strongly ossified and densely denticulated along its length (Fig. 5). Postorbital length is longer

than that of other studied populations. The PrOL/PsOL ratio ranged from 0.69 to 0.84 ($\bar{x}$ =0.77). In adult specimens, the lower lip is thick with a retracted medial lobe revealing the dentary. The ethmoid bone is narrower than its length. Vertebrae 41-43 ($\bar{x}$ =42.1, n=12), Gill Rakers (GR) 13-15 ($\bar{x}$ =14.1 Median=14).

DESCRIPTION: D IV 8, A III 5, P I 15-16, V I 8, C 18; LL 41-47 ($\bar{x}$ =43.7, Median=44), RSA 7.5-8.5 ($\bar{x}$ =7.55, Median=7.5), RSB 4.5-6.5 ($\bar{x}$=5.47, Median=5.5). Pharyngeal teeth in adults 4.3.2/4.3.2., GR 13-15 ($\bar{x}$= 14.1 X=14), VE 41-43 ($\bar{x}$= 42.1, n=12). A medium-sized species, rarely reaching 500 mm. The body is elongated relative to maximum body depth and compared to other *Luciobarbus* species. The head is large with respect to the body with head length 22-28% of SL. Infraorbital bones are narrow. The first barbel did not reach the anterior edge of the eye, but, in some specimens, barbels reached the rim of the eye and extended to half the width of the eye. The second barbel usually extended beyond the posterior rim of the eye, rarely reaching the preopercle. The anterior barbel is 19.3-33.3%, and the second 21.2-41%, of HL. The lips are thick with the inferior usually retracted in adults revealing the dentary bone. In some specimens, lips are not retracted and exhibited a well-developed medial lobe. The lacrimal bone has a medium-sized manubrium. The snout is prominent, marked in some specimens, with preorbital length 7.8-10.3% of SL; postorbital length ranged from 9-13.6% SL. The iris, as in other Moroccan species of *Luciobarbus* is yellow pigmented at the superior border but is less conspicuous than in other species. The jugal space closes at the same plane of the vertical of the eye, and 11-12 pores were present in the inferior branch of the pre-operculum. The dorsal fin is posterior on the body with the predorsal distance of 48.2-55.8% SL. The profile of the dorsal fin is straight or slightly concave, with the last single ray ossified and profusely denticulated (Fig. 12). The caudal peduncle is slightly more elongated than that in Rifian populations, with a height of 30-39.3% SL. The height of the caudal peduncle is 1.4 to 1.98 times the length of the anal peduncle. The pectoral and ventral fins are longer in males, and the anal fin is longer in the

females. Males exhibit nuptial tubercles in the preorbital space. Ventral fins are inserted forward to dorsal fin insertion. The caudal fin is 16.6-27.3% SL. Morphometric and meristic measurements for the holotype and paratypes of *Luciobarbus maghrebensis* are represented in Table 8. The coloration of *L. maghrebensis* is silver-yellowish with darker fins (Fig. 11). Some specimens exhibit a darker longitudinal band in the center of the body. Juveniles present blotches, as in other *Luciobarbus* species. The skull is wide with a narrow ethmoid bone and a large opercle; the pharyngeal bone is wide with a long inferior process. The lacrimal bone is well developed, and infraorbital bones are narrow. The dentary has a long anterior process, and the maxilla has a small palatine process. The basioccipital plate is wide and triangular.

ETYMOLOGY: The species name *"maghrebensis"* has been selected because it is mainly distributed in the northwestern area of the Maghreb region.

DISTRIBUTION: This species is endemic to north-central Morocco, inhabiting Sebou Basin and rivers flowing into the Moulay Bouselham Lagoon on the Atlantic slope (Fig. 1).

COMMON NAME: We propose using the English common name "Maghreb barbel" for this new species.

HABITAT AND BIOLOGY: The species can inhabit rivers of varying typology within its distribution range. Upstream it is substituted for the trout, and in the lower courses of rivers and calm currents it is locally highly abundant. From April to May the species migrates upstream to headwaters for spawning. The species is also present in reservoirs.

CONSERVATION: *Luciobarbus maghrebensis* spawning migration has been affected by dams, which present physical barriers to upstream migration. The presence of exotic species in reservoirs, as well as poor water quality in the lower courses, due to fertilizers and pesticides, have probably been primary causes of the decline in populations in recent years. No quantification of the decline in numbers is available, but the species is still abundant and thriving locally. We suggest that this species should be included in the IUCN category of Least Concern (LC)

GENETICS: Uncorrected genetic distance of mitochondrial gene cyt*b* between *Luciobarbus maghrebensis* and the other analyzed species are represented in Table 7. *L. maghrebensis* shows 12 diagnostic positions in the cyt*b* gene. A previous allozyme study found three diagnostic loci between *L maghrebensis* (GP1-2*, GP15* and PGDH-1*) and Grou population (Machordom *et al.*, 1998).

Fig. 8.— Holotype of Luciobarbus rifensis from Laou river, Laou Basin, in Derdara, Morocco. MNCN 290.641.

Luciobarbus rifensis Doadrio, Casal-Lopez & Yahyaoui, **sp. nov.**
urn:lsid:zoobank.org:act:F32CCBDD-7C77-4584-889E-1DAA0733AF43

TYPE MATERIAL: Holotype: Fig. 8, Table 9. MNCN 290.642; female, 177.2 mm (SL); Laou River, Laou Basin, Derdara, Chefchauen Province, Mediterranean slope in Morocco; (35.118986-5.288900) (Fig. 1); 6/6/2013. Coll: Doadrio, I; Yahyaoui, A; Casal-Lopez, M; Perea, S.

Paratypes: Table 9. MNCN 290.639-641, 290.643-652, 290.655, 290.657-663, 290.665-667: Twenty-four specimens from Laou River, Laou Basin, Derdara, Chefchaouen Province, Mediterranean slope in Morocco; (35.118986, -5.288900); 6/6/2013; Coll: Doadrio, I; Yahyaoui, A; Casal-Lopez, M.; Perea, S. MNCN 284.939-940, 284.942-945, 284.947-948, 284.950-951, 284.953-964: Twenty-two specimens from Laou River, Laou Basin, Beni Ferten, Tétouan Province, Mediterranean slope in Morocco (35.353254, -5.184840); 8/4/2007. Coll: Doadrio, I; Doadrio I jr; Perea, S. MNCN 280.162-163, 280.165, 280.168, 280.170, 280.172-174, 280.176-181, 280.183, 280.185-186; 290.671-696: Forty-three specimens from Loukos River, Loukos Basin, Souk Had, Laghdir, Chefchaouen Province, Atlantic

slope in Morocco (35.02624, -5.404660) 30/10/2011. Coll: Doadrio, I; Yahyaoui, A; Casal-Lopez, M; Garzon, P. MNCN 290.707-714, 290.716-722, 290.725-731. Twenty-two specimens from Hachef River, Hachef Basin, Dar Chaoui, Tétouan Province, Atlantic slope in Morocco (35.526763, -5.713771); 6/6/2013. Coll: Doadrio, I; Yahyaoui, A; Casal-Lopez, M; Perea, S.

Table 9.— Morphometric and meristic measurements of holotype and paratype series of Luciobarbus rifensis.

Morphometric measurement	Holotype MNCN 290642	Paratypes n=111		
	Measurement (mm)	Range	Mean	Standard Deviation
SL	177.2	77.2-226	127.2	41.6
PrDD	90.4	41.4-122.8	69.4	22.5
PrPD	45.3	20.9-63.3	34.1	11.4
PrVD	94.6	43.4-126.2	71.2	23.1
PrAD	135.3	58.4-173.4	98.4	32.1
PVL	50.7	21.9-65.4	37.5	12.0
CPL	68.6	26.9-86.0	46.7	15.9
APL	33.2	13.2-43.5	23.6	8.3
BD	45.4	18.4-58.2	32.5	10.6
BLD	19.1	8.4-21.8	13.6	3.7
HL	41.1	19.3-56.7	31.9	10.1
PrOL	15.7	6-20.3	11.2	3.5
ED	7.0	3.7-11.4	6.6	1.9
PsOL	18.6	8-27.1	14.4	5.0
B1L	8.1	3.0-16.3	7.6	3.3
B2L	13.3	4.1-22	10.0	4.2
PFL	28.4	12.3-42.2	22.3	7.5
VFL	23.9	11.1-32.3	18.5	5.5
DFL	23.2	9.5-28.7	16.1	4.8
DFH	31.9	11.2-41.9	21.7	7.1
AFL	12.5	4.8-17.3	9.4	3.1
AFH	31.1	11.7-42.1	20.8	7.7
CFL	36.7	16.2-49.2	26.9	8.3
LL	44	42-46	43.8	0.8
RSA	9.5	8.5-9.5	8.7	0.4
RSB	5.5	3.5-5.5	4.7	0.4

Holotype and a series of paratypes (112 specimens) have been deposited in the Fish Collection of the Museo Nacional de Ciencias Naturales, Madrid, Spain.

DIAGNOSIS: Differs from other known species of *Luciobarbus* by the following combination of characters: 42-46 scales on the lateral line ($\bar{x}$=43.9, Median=44), 8.5-9.5 scales above lateral line ($\bar{x}$=8.8, Median= 8.5), and 3.5-5.5 scales below lateral line ($\bar{x}$=4.7, Median= 4.5). Insertions of dorsal fin

and ventral fin were situated similarly on the body or the insertion of the dorsal fin is slightly anterior to the ventral fin origin. Last single fin ray is ossified and denticulated along its length. The denticulations at mid-height of the ray are shorter than the ray width. In adult specimens, the lower lip is well developed with retracted medial lobe revealing the dentary. The ethmoid bone is wider than long. Vertebrae 41-42, ($\bar{x}$=42, n=12); gill rakers 10-15 ($\bar{x}$=13.6 Median=13).

DESCRIPTION: D IV 8, A III 5, P I 15-16, V I 8, C 18. LL 42-46 (X=43.9, X=44); RSA 8.5-9.5 ($\bar{x}$=8.8, Median=8.5); RSB 3.5-5.5 ($\bar{x}$=4.7, Median=4.5). Pharyngeal teeth in adults 4.3.2/4.3.2. GR 10-15 ($\bar{x}$=13.6 Median=13); VE 41-42 ($\bar{x}$=42 n=12). A medium-sized species that rarely reaches 500 mm. The body is short and deep in comparison with other *Luciobarbus* species with maximum body depth 22-28% SL. The head is large with respect to the body with head length 23.2-27.8% SL. Circumorbital bones are narrow. The barbels were long with the first barbel not reaching the anterior edge of the eye. The second barbel usually extended to the posterior edge of the eye, but did not reach the preopercle. In females, the first barbel is 14.5-36% HL and the second 20-40.3% HL. The lips are thick with the inferior usually retracted in adults making the dentary bone visible. Some specimens do not exhibit inferior lip retraction and show well developed lips (Fig. 8). The lacrimal bone has a short manubrium. The eye is placed anteriorly in the head; preorbital length is 7.1-10.6% SL, and postorbital length is 10.2-12.5% SL. The snout is characteristically rounded. The iris, as in other Moroccan species of *Luciobarbus,* is yellow pigmented at the superior border but is less conspicuous than in other species. The jugal spacious closes at the same vertical plane of the eye and from 11 to 12 pores were present in the inferior branch of the preopercular. The dorsal fin was located posterior on the body, with the predorsal distance being 51.2-55.9% SL. The profile of the dorsal fin was slightly concave, and presents the last single ray strongly ossified and denticulated. The caudal peduncle (CPL) is high and short with a height of 32.6-39% SL. The height of the caudal peduncle is 1.2 to 2 times the length of the anal peduncle. The pectoral and

ventral fins are longer in males, and the anal fin is longer in females. Males have nuptial tubercles in the preorbital space, but not as well developed as in other *Luciobarbus* species. Ventral fins are inserted in the same vertical plane as the origin of the dorsal fin or slightly posterior to the dorsal fin. The length of the caudal fin is 14.9-26.5% SL. Morphometric and meristic measurements for the holotype and the paratypes of the newly described species are represented in Table 9. The coloration of *L. rifensis* is brown-yellowish with darker fins, with the body becoming progressively paler in the ventral region (Fig. 7). Juveniles present black blotches as in other *Luciobarbus* species. The skull is long and narrow with a wide ethmoid bone. Infraorbitals are narrow with a large lacrimal bone. The basioccipital plate is triangular. The palatine process of maxilla is small. The superior branch of the pharyngeal bone forms a closed angle with respect to inferior branch.

Fig. 8. Well developed lips with medial lobe in a specimen of Luciobarbus rifensis from the Loukos River on the left and lips retracted revealing dentary in one specimen of Luciobarbus rifensis from Laou River on the right

ETYMOLOGY: The species name "*rifensis"* has been selected because the distribution range comprises the Rifian Mountains of Morocco.

DISTRIBUTION: The new species is endemic to north Morocco, inhabiting waters from the Loukos Basin on the western Atlantic slope to the Laou Basin on the eastern Mediterranean slope (Fig. 1).

COMMON NAME: We propose the English common name "Rifian barbel" for this newly identified species.

HABITAT AND BIOLOGY: The species is ubiquitous and inhabits streams and rivers with substrata ranging from sandy to stony, being absent only in small streams near the sources of rivers and in shallow waters. The species is present in reservoirs. The spawning period is variable, but usually takes

place in April and May. The species migrates upstream to spawn in cold and oxygenated waters where the females excavate a nest in the gravel.

CONSERVATION: *Luciobarbus rifensis* is a common species. However, its distribution area has been extensively transformed in recent years due to construction of dams, which also hamper upstream migration during the spawning period. In addition, reservoirs can harbor exotic species, some piscivorous, which could be a potential threat to barbels. The area where the species occurs has an increasing interest for tourism activities, which increases water abstraction for recreational use, leading to decreased water levels in summer as well as water oxygen depletion. The species is currently common and locally abundant. For this reason, we suggest that it should be included in the IUCN category as Least Concern (LC).

GENETICS: Uncorrected genetic distance based on mitochondrial gene cyt*b* between *Luciobarbus rifensis* populations and the other analyzed populations are represented in Table 7. *L. rifensis* shows 6 diagnostic positions in the cyt*b* gene. An earlier allozyme study found four diagnostic loci to *L. rifensis* (MPI, GPI-5, IDHP-2 and CK) with respect to Sebou populations (Machodom *et al.*, 1998).

Fig. 10.— Holotype of Luciobarbus rabatensis from Grou River, Bou Regreg Basin, Sebt Ait Rahhou, (Morocco). MNCN 71920.

Luciobarbus rabatensis Doadrio, Perea & Yahyaoui, **sp. nov.**

urn:lsid:zoobank.org:act:45CC4380-B532-4AEC-9050-692042FF0365

TYPE MATERIAL: Holotype: Fig. 10. Table 10. MNCN 71920, male, 129.9 mm (SL); Grou River, Bou Regreg Basin, Sebt Ait Rahhou, Atlantic slope of Morocco (33.164678, -6.366963) (Fig. 1). Coll: P. Garzón, I. Doadrio, and A. Yahyaoui. 17/04/1991.

Paratypes: MNCN 71725-746, 71918-919, 71921-928; Thirty-two specimens from the Grou River, Bou Regreg Basin, Sebt Ait Rahhou (33.164678, -6.366963) Morocco. Coll: P. Garzón, I. Doadrio and A. Yahyaoui. 17/04/1991. Holotype and the series of paratypes (33 specimens) have been deposited inthe Fish Collection of the Museo Nacional de Ciencias Naturales (Madrid, Spain).

Table 9.— Morphometric and meristic measurements of holotype and paratypes of *Luciobarbus rabatensis*. Spain).

Morphometric measurementes	**Holotype MNCN 71920**	**Paratypes n=32**		
	Measurement in mm	**Range**	**Mean**	**Standard Deviation**
SL	129.9	53.1-113.6	74.8	14.5
PrDD	66.1	29.1-59.9	40.4	7.3
PrPD	33.8	14.4-30.4	19.5	4.3
PrVD	68.2	28.8-60.7	39.8	8
PrAD	96.5	39.1-84.5	55.5	10.8
PVL	34.7	14.3-30.8	20.8	4
CPL	49.5	18.9-40.5	26.8	5.4
APL	24.7	9.1-21.7	13.8	2.8
BD	31.7	14.9-28.8	16.7	3.5
BLD	15.2	6.3-14.1	8.9	1.8
HL	32.1	14.2-27.3	18.8	3.4
PrOL	12.8	4.1_11-1	6.3	1.6
ED	6.3	3.5-5.6	4.4	0.6
PsOL	16.3	6.6-13.7	9.2	1.7
B1L	8.2	3.1-6.7	4.6	0.8
B2L	9.9	4.1-8.4	5.7	1.1
PFL	25.8	11.4-22.5	15.4	2.7
VFL	21.6	9.2-18.6	12.7	2.2
DFL	16.5	7.4-15	10.1	1.9
DFH	24.5	10.3-21.9	14.3	2.8
AFL	9.3	3.7-8.1	5.2	1.1
AFH	24.3	10-21.1	13.9	2.5
CFL	26.5	12.7-24	17.1	2.7
LL	46	45-50	46.5	1.3
RSA	9.5	8.5-10.5	9.6	0.4
RSB	6.5	6.5-7.5	6.7	0.4

DIAGNOSIS: Differs from other known species of *Luciobarbus* by the following combination of characters: Number of scales on LL 45-50 ($\bar{x}$=46.5, Median=46); number of scales above lateral line 8.5-10.5 ($\bar{x}$=9.6, Median=

9.6). Number of scales below lateral line 6.6-7-5 ($\bar{x}$=6.7, Median=6.5). Insertion of dorsal and ventral fins is in the same vertical plane. Last single fin ray is ossified, with deep denticulations along its length. Denticulations at mid-height of the ray are equal to or longer than ray width. In adult specimens, the inferior lip is retracted revealing the dentary. The ethmoid bone is wider than its length. The maxilla showed a large palatine process. Vertebrae 41-42 ($\bar{x}$=42, n=6); gill rakers 14-12 ($\bar{x}$=12.9). Diagnostic characters of the analyzed *Luciobarbus* populations are presented in Table 11.

DESCRIPTION: D IV 8, A III 5, P I 15-16, V I 8, C 18; LL 46-50 ($\bar{x}$= 46.5, Median=46), RSA 8.5-10.5 ($\bar{x}$=9.6, Median=9.6), RSB 6.5-7.5 ($\bar{x}$=6.7, Median=6.5). Pharyngeal teeth in adults 4.3.2/4.3.2. GR 12-14 ($\bar{x}$=12.9 Median=13). VE 41-42 ($\bar{x}$=42 n=6). It is a medium-sized species that rarely reaches 500 mm. The body is slightly more elongated in comparison with *L. rifensis* and *L. maghrebensis* with maximum body depth ranging from 24.4-28.5% SL. The head is large relative to the body, similar to other *Luciobarbus* species, with head length 23.5-26.8 % SL. The preorbital distance is short and the proportion with respect to head length is 29.2-40.5% SL. The circumorbitals bones are narrow. The barbels are similar in size to those of *L. maghrebensis* and *L. rifensis*, but, due to a shorter snout, the first barbel usually reaches the anterior edge of the eye. The second barbel usually extends to the posterior edge of the eye, but does not reach the preopercle. The anterior barbel is 22.1-26.3% HL and the second 22.3-32.8% HL. The lips of adult individuals are usually retracted, and the dentary bone is visible. The lacrimal bone has a short and high manubrium. As in other Moroccan *Luciobarbus* species, the iris is yellow pigmented at the superior border. The dorsal fin is placed posteriorly on the body with a predorsal lenght of 50.9-56.3% SL. The profile of the dorsal fin is concave with the last single ray ossified with pronounced denticulation. The caudal

peduncle (CPL) is slightly more elongated than in *L. maghrebensis* and *L. rifensis*, with a height form 34.7 to 38% SL. The height of the caudal peduncle is 1.4 to 1.7 times the anal peduncle length. The pectoral and ventral fins are longer in males, and the anal fin is longer in females. Males present nuptial tubercles in the preorbital space. The caudal fin is long, with a length of 20.4-24.2% SL. The colour is slightly yellow, brownish in the dorsal region and more silver in the ventral area. The skull is long and narrow with the ethmoid bone wider than its length. The lacrimal bone is short. The basioccipital bone has a wide pentagonal plate. The palatine process of the maxilla is more conspicuous and the pharyngeal bone is narrower than in specimens of the other populations studied.

ETYMOLOGY: The species name *"rabatensis"* has been selected because its distribution area mainly comprises the Bou Regreg Basin, which flows through Rabat City.

DISTRIBUTION: This new species is endemic to north Morocco, inhabiting the Bou Regreg Basin (Fig. 1).

COMMON NAME: We propose the English common name "Rabat barbel" for this species.

HABITAT AND BIOLOGY: As other barbels of Morocco, with the exception of *L. magniatlantis*, *L. rabatensis* inhabits rivers of differing typologies. The species is present in reservoirs. Spawning takes places at the end of April and the beginning of May. At that time, individuals migrate upstream for spawning in cold and oxygenated waters where the females excavate a nest in the gravel.

CONSERVATION: The habitat of *Luciobarbus rabatensis* has been extensively transformed with the growth of Rabat City, urban development, water usage, increased pollution linked to agriculture, construction of dams and reservoirs, and introduction of exotic species. Although, an abundant population of this species is still found, it is suffering a slight decline. For this reason, we suggest that this species should be included in the IUCN category of Near Threatened (NT).

GENETICS: Uncorrected genetic distance between *Luciobarbus rabatensis*

and the sister population of *Luciobarbus ksibi* to mitochondrial gene cyt*b* was 1.7%. *Luciobarbus rabatensis* shows 6 diagnostic positions in the cyt*b* gene. A previous allozyme study found one diagnostic locus (*GPI-2* 118*) with respect to other African *Luciobarbus*.

Supplementary material

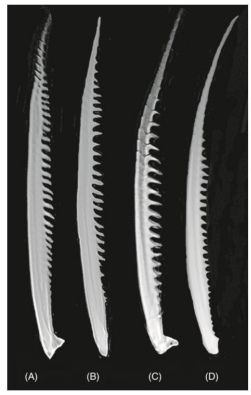

Fig.1S.— Last single dorsal fin ray in adult specimens (SL>120 mm) of the studied populations. A: Rifian population (Laou River). B: Sebou Basin (Ifrane River). C: Bou Regreg (Grou River). D: Luciobarbus ksibi (Kasab River).

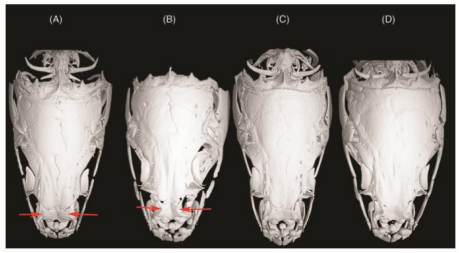

Fig.2S.Dorsal view of the skull of the populations under study. Arrows indicate width of the ethmoid bone. A: Rifian population (Laou River) B: Sebou Basin (Ifrane River) C: Bou Regreg (Grou River), D: Luciobarbus ksibi (Kasab River).

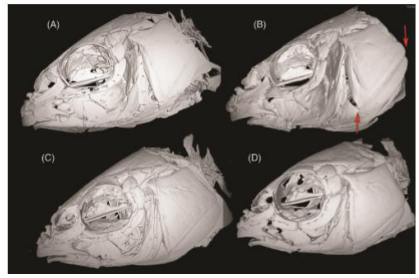

Fig.3S. Lateral view of the skull of the studied populations. Arrows show the length of the opercular bone. A: Rifian population (Laou River). B: Sebou Basin (Ifrane River). C: Bou Regreg (Grou River). D: Luciobarbus ksibi (Kasab River).

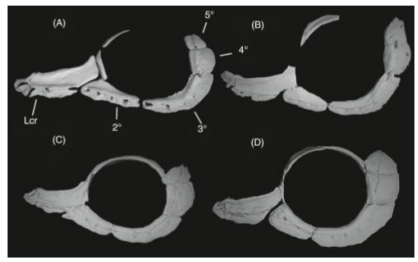

Fig.4S.— Infraorbital bones of the studied populations. A: Rifian population (Laou River). B: Sebou Basin (Ifrane River). C: Bou Regreg (Grou River). D: Luciobarbus ksibi (Kasab River). Lcr = Lacrymal. 2°-5°: Infraorbitals.

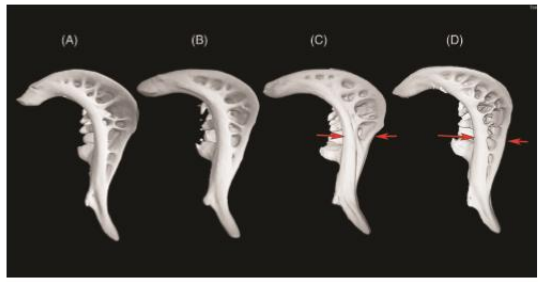

Fig.5S. Pharyngeal teeth of the studied populations. The arrows show the width of the pharyngeal bone. A: Rifian population (Laou River). B: Sebou Basin (Ifrane River). C: Bou Regreg (Grou River). D: Luciobarbus ksibi (Kasab River).

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3. CHAPTER II

This chapter is based upon the manuscript:

Doadrio, I., Casal-López, M., Perea, S., & Yahyaoui, A. (2016). Taxonomy of rheophilic Luciobarbus Heckel, 1842 (Actinopterygii, Cyprinidae) from Morocco with the description of two new species. **Graellsia, 72(1), e039.**

TAXONOMY OF RHEOPHILIC *LUCIOBARBUS* HECKEL, 1842 (ACTINOPTERYGII, CYPRINIDAE) FROM MOROCCO WITH THE DESCRIPTION OF TWO NEW SPECIES

ABSTRACT

The genus *Luciobarbus* Heckel, 1843 is characterized by medium to large fish species occurring in large rivers and downstream zones with slow-moving waters. Remarkably also rheophilic *Luciobarbus* occur in Morocco, which are of small size and exhibit distinct morphological traits as well as different habitat requirements. These rheophilic species have traditionally been assigned to *Luciobarbus nasus* (Günther, 1874) and *L. magniatlantis* (Pellegrin, 1919), although some authors consider *L. magniatlantis* as a junior synonym of *L. nasus*. This lack of consensus on the taxonomy of rheophilic barbs is constrained by limited population studies that do not encompass their entire distribution range. Using molecular, morphometric, and osteological data we studied populations of rheophilic barbs of three drainage basins in which they are currently present: Tensift, Moulouya and Oum er Rbia. Our results clearly identified the rheophilic barbs of each basin as different species. The species *Luciobarbus magniatlantis* is a well-recognized species endemic to Tensift Basin. In contrast, the investigated populations from the Moulouya and Oum er Rbia basins could not be assigned to any previously described species. Consequently, we describe two new *Luciobarbus* species in the Moulouya and Oum er Rbia basins.

Introduction

The genus *Luciobarbus* Heckel, 1843 comprises more than 30 species of medium to large size widely distributed along rivers draining into the Persian Gulf and the Mediterranean, Caspian, and Black seas (Doadrio, 1994, Kottelat & Freyhof, 2007). With approximately 20 species, North Africa and the Iberian Peninsula show the highest diversity in *Luciobarbus* populations, with the genus being one of the main component of the freshwater fish fauna of these regions (Doadrio, 1994; Machordom *et al.*, 1995). Phylogenetic studies using morphological characters, have provided evidence for *Luciobarbus* monophyly (Doadrio, 1990), but monophyly has not been unambiguously retrieved by molecular markers, due to the position of the genus *Capoeta* within *Luciobarbus* (Levin *et al.*, 2012; Yang *et al.*, 2015). The genera *Capoeta* and *Luciobarbus* comprise limnetic species, most of which migrate upstream to spawning areas, characterized by the presence of gravel and clear waters (Banarescu, 1999; Doadrio *et al.*, 2011). Although they can survive in a variety of habitats, outside of the reproductive period they are most abundant in downstream areas. Remarkably, in the freshwaters of Morocco rheophilic *Luciobarbus* species can also be found, which have habitat requirements and morphological traits that differ from other *Luciobarbus* species (Doadrio, 1990).

The rheophilic species of *Luciobarbus* are sympatric with limnetic *Luciobarbus* and *Carasobarbus* species in rivers of the Atlas Mountains in Morocco (Doadrio, 1994), but their habitat is restricted to riffle areas, while other Atlas Mountain *Luciobarbus* are more widespread, with preference for slower waters (Doadrio 1990). The rheophilic *Luciobarbus* in Morocco have been assigned, so far, to two species: *Luciobarbus nasus* (Günther, 1874) and *Luciobarbus magniatlantis* (Pellegrin, 1919), which are morphologically characterized, with respect to other *Luciobarbus* species, by size rarely reaching 20 cm standard length; thick barbels placed at the extreme

anterior of the snout; and males with equal-sized nuptial tubercles uniformly distributed over the body (Almaça, 1970; Doadrio, 1990). This difference in morphology placed *L. nasus* and *L. magniatlantis* in an unresolved taxonomic position, and they could not be assigned to *Luciobarbus* when the genus was formally defined on the basis of osteological traits (Doadrio, 1990). Subsequently, molecular studies of mitochondrial genes have unambiguously placed the rheophilic *L. nasus* in the genus *Luciobarbus* together with other North African species (Machordom & Doadrio, 2001a,b).

Within rheophilic *Luciobarbus* from Morocco, the taxonomic position of *L. magniatlantis* remains unclear. This species has been traditionally considered a junior synonym of *L. nasus* (Esteve, 1947; Leveque & Daget, 1984). However, several authors reported morphological traits that unequivocally separate the rheophilic *Luciobarbus* into two morphological types and consider *L. magniatlantis* as a separate species (Pellegrin, 1939; Almaça, 1970, 1971; Doadrio, 1990).

So far there were no available studies on the population structure of the rheophilic barbs of the four Moroccan basins in which they occur: Moulouya Basin, on the Mediterranean slope, and Oum er Rbia, Tensift, and Kasab basins, on the Atlantic slope (Almaça, 1970; Doadrio, 1994; Borkenhagen & Krupp, 2013). This lack of population studies compounds confusion about the type locality of *Luciobarbus nasus* and has hindered the taxonomic work. While the type locality, Tensift Basin, of *L. magniatlantis* is not in question (Pellegrin, 1919), the type locality of *L. nasus* is unclear, because the two syntype specimens were preserved together with sea fishes from Mogador (Essaouira) on the seacoast of Morocco (Günther, 1874). It is probable that the two syntypes of *Barbus nasus* came from material collected in the Kasab River near Mogador by K. v. Fritsch and J. Rein in 1872 (Borkenhagen & Krupp, 2013).

We review the taxonomy of rheophilic *Luciobarbus* from Morocco, investigating morphology and genetic traits of specimens from Moulouya, Tensift, and Oum er Rbia basins, where they have been previously reported

(Almaça, 1970). The recognition of two new distinct taxonomic entities, lead us to the description of two new species of *Luciobarbus* for the freshwaters of Morocco.

Material and Methods

Our study of *Luciobarbus* populations was based on 15 specimens (14 males, one female) from Tensift Basin, 33 specimens (21 males, 12 females) from Oum er Rbia Basin, and 12 specimens (12 males) from Moulouya Basin (Fig. 1; Table 1). Material from Kasab Basin could not be collected despite consecutive efforts over the course of several years. The Kasab River has been intensively modified in recent years by anthropogenic pressure as a consequence of the touristic development of Essaouira (Mogador). Three cyprinid species were collected in the Kasab River during the past two decades: *Luciobarbus ksibi* (Boulenger, 1905), *"Labeobarbus" reinii* (Günther, 1874), and *Carasobarbus fritschii* (Günther, 1874). However, the endemic *"L". reinii* and *L. nasus* from the Kasab River have not been found in the past ten years, and it is likely that the species have disappeared or at least they are extremely rare. Therefore, our study of rheophilic *Luciobarbus* from the Kasab River was limited to the museum specimens of the two syntypes of *Barbus nasus*.

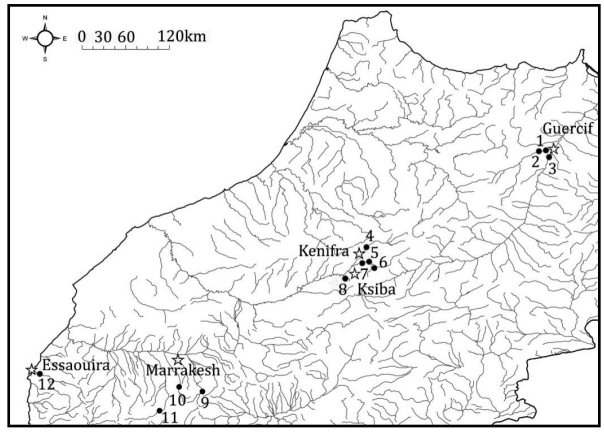

Fig. 1. Sampling localities of Luciobarbus populations in Morocco. Melloul River, Guercif (1, 2); Moulouya R., Ghafoula (3); Oum er Rbia R., El Borj (4); Chbouka R., El Herri (5); Douna R., El Khemis (6); Serrou R., El Herri (7); Arba R., Ksiba (8); Ourika R., (9); Reraia R., Moulay Brahim (10); N'Fiss R., Kasbah Goundafi, and Imaounane (11); Kasab R., Essaouira (Former Mogador) (12).

The material studied comprised the following specimens and locations: *Tensift Basin*: 3 specimens from the Reraia River, Moulay Brahim (31.2862, -7.9597), Morocco (voucher numbers: MNCN 280454-280456); 2 specimens from the N'Fiss River, Imaounane (30.9899, -8.2011), Morocco (voucher numbers: MNCN 208172-280173); 7 specimens from the Ourika River, Morocco (voucher numbers: MNHN-IC-1919-0431-0437); Lectotype and Paralectotypes of *Barbus magniatlantis* Pellegrin, 1919; 3 specimens from N'Fiss River, Kasbah Goundafi (30.9899, -8.2011), Morocco (voucher numbers: MNHN-IC-1919-0438-040). *Oum er Rbia Basin*: 1 specimen from the Douna River, El Khemis (32.7508, -5.5416) Morocco, (voucher number: MNCN 55094); 2 specimens from the Serrou River, El Herri (32.8276, -5.6152), Morocco (voucher numbers: MNCN 208115-280116); 14 specimens from Arba River, Ksiba (32.5668, -6.0174), Morocco (voucher numbers: MNCN: 54987-55000); 9 specimens from the Oum er Rbia River, El Borj (33.0157, -5.6295), Morocco (voucher numbers: MNCN 208168-208169; MNCN 71972-71978); 7 specimens from the Chbouka River, El Herri (32.8595, -5.6213), Morocco (voucher numbers: MNCN 279704-279707; MNCN 280083; MNCN 279695-279696). *Moulouya Basin*: 3

specimens from the Melloulou River, Guercif (34.2180, -3.3467 and 34.215, -3.3756), Morocco (voucher numbers: MNCN 286595-286596; MNCN-290831); 9 specimens from the Moulouya River, Ghafoula (34.145, -3.388), Morocco (voucher numbers: MNCN 290832-290840). *Kasab Basin*: 2 syntype specimens of *Barbus nasus* Günther, 1874 from the Kasab River preserved at the Natural History Museum (BMNH 1874.1.30.25-26).

All sampling sites (Fig. 1) presented similar riverine morphology, with clear water, rapid current and gravel bottom.

Twenty-four morphometric measurements were taken with digital callipers (0.01 mm), and ten meristic variables were counted with a stereoscopic microscope. The following acronyms were used for morphometric and meristic characters: A, number of anal fin rays; AFH, anal fin height; AFL, anal fin length; APL, anal peduncle length; BL1, first barbel length; BL2, second barbel length; BD, body depth; BLD, body least depth; C, central caudal fin rays; CFL, caudal fin length; CPL, caudal peduncle length; D, dorsal fin rays, DFL dorsal fin length; DFH dorsal fin height; ED, eye diameter; GR, gill rakers (number); HL, head length; IOW, interorbital width; LL lateral line scales; P, pectoral fin rays; PFL, pectoral fin length; PrAD, pre-anal distance; PrDD, pre-dorsal distance; PrOL, pre-orbital length; PrPD, pre-pectoral distance; PrVD, pre-ventral distance; PsOL, postorbital length; PVL, pectoral-ventral length; RSA, scale rows above lateral line; RSB scale rows below lateral line; SL, standard length; V, ventral fin rays; VFL, ventral fin length; VE, Number of vertebrae. The number of vertebrae was obtained by counting on X-ray images of specimens from all sampled populations. Osteological characteristics were investigated through computer tomography (CT) scan and digital dissection using VGStudio MAX v2.2 (Volume Graphics, http://www.volumegraphics.com).

After constructing the measurement matrix, Burnaby's method was used to correct for size effect. The Burnaby method removes the effects of a within-population size-factor from between-group morphometric analyses through an orthogonal projection procedure (Burnaby, 1966; Röhlf &

Bookstein, 1987).

All analyses were conducted with the corrected matrix. Morphometric and meristic characters were analysed independently. To assess sexual dimorphism and to identify the variables that contributed most to the variation among populations, two principal component analyses (PCA) were performed using the covariance matrix for morphometric characters. Statistical analyses were carried out using PAST software (Hammer *et al.*, 2001).

For molecular analyses, we obtained specimens of rheophilic *Luciobarbus* from Oum er Rbia, Tensift, and Moulouya basins along with the limnophilic *L. ksibi* from Oum er Rbia, Tensift and Kasab basins, (Table 1). Also, the Iberian species of *Luciobarbus* and *L. setivimensis* (Valenciennes, 1842) from Algeria were added. *Aulopyge hueguelli* Heckel, 1843 and *Barbus meridionalis* Risso, 1827 were selected as outgroups, based on previous phylogenetic analyses (Zardoya & Doadrio, 1999). Total genomic DNA was extracted from fin-clip tissue using the commercial kit Biosprint15 for tissue and blood (Qiagen). For each specimen, the complete region (1140 bp) of the mitochondrial cytochrome b (cytb) was amplified. Primers and protocols used for PCR for cytb followed Machordom *et al.* (2001 b). After checking PCR products on 1% agarose gels, they were purified by ExoSAP-IT™ (USB) and directly sequenced on MACROGEN service using a 3730XL DNA sequencer. Sequences were deposited in the GenBank database under accession numbers KU257523-KU257539 .

Phylogenetic analyses were performed using Bayesian inference (BI) implemented in MrBayes v. 3.2 (Ronquist *et al.*, 2012). The Akaike Information Criterion (Akaike, 1973) implemented in jModeltest (Posada, 2008) was used to determine the evolutionary model that best fit the data. In this case, TIM1+G model was selected Bayesian inference was performed using two independent runs of four Markov Montecarlo coupled chains (MCMC) of 5×10^6 generations each to estimate the posterior probability distribution. Topologies were sampled every 100 generations, and a

majority-rule consensus tree was estimated after discarding the first 10% of generations. The robustness of the clades was assessed using Bayesian posterior probabilities. The average uncorrected p-distances among *Luciobarbus* populations were calculated for the cytb gene using MEGA package v. 6.0 (Tamura *et al.*, 2013) .

Table 1.— Sampling localities for *Luciobarbus* from Moroccan and GenBank Accession numbers.

Population assignment/species	Locality	No. Individuals studied Morphology/molecular	Number in Phylogenetic tree	GenBank Accession Numbers	Number in map
Moulouya population	Melloulou R. Guercif/ Moulouya Basin	3/1	M3	KU257527	1, 2
Moulouya population	Moulouya R. Ghafoula/ Moulouya Basin	9/3	M1, M2, M4	KU257525, KU257526, KU257528	3
Oum er Rbia population	Oum er Rbia R. El Borj/Oum er Rbia Basin	9/3	R1, R2, R3	AY004744, KU257531, KU257532	4
Oum er Rbia population	Chbouka R. El Herri/Oum er Rbia Basin	7/-			5
Oum er Rbia population	Douna R. El Khemis/Oum er Rbia Basin	1/-			6
Oum er Rbia population	Serrou R. El Herri/Oum er Rbia Basin	2/1	R4	KU257533	7
Oum er Rbia population	Arba R. Ksiba/Oum er Rbia Basin	14/-			8
Tensift population. Lectotype and Paralectotypes of *Barbus magniatlantis*	Ourika R./Tensift Basin	7/-		xxx	9
Tensift population	Reraia R. Moulay Brahim/ Tensift Basin	3/2	T2, T3	KU257536, KU257537	10
Tensift population. Paralectotypes of *Barbus magniatlantis*	N'Fiss R. Kasbah Goudafi/ Tensift Basin	3/-		xxx	11
Tensift population	N'Fiss R. Imaounane/Tensift Basin	2/2	T1, T4	KU257534, KU257535	11
Kasab population. Syntypes of *Barbus nasus*	Kasab R. Essaouira/Kasab Basin	2/-			12
L. ksibi	Kasab R. /Kasab Basin.	-/2	1, 2	KU257523, KU257524	12
L. ksibi	Reraia R./Tensift basin	-/2	3, 4	KU257538, KU257539	10
L. ksibi	Chbouka R./Oum er Rbia basin	-/2	5, 6	KU257529, KU257530	5
L. bocagei	Duratón R./Duero Basin	-/1			
L. comizo	Tajo R./Tajo Basin	-/1			
L. graellsii	Irati R./Ebro Basin	-/1			
L. guiraonis	Turia R./Turia Basin	-/1			
L. microcephalus	Zujar R./Guadiana Basin	/1			
L. sclateri	Segura R./Segura Basin	/1			
L. setivimensis	Soumman R./Soumman Basin	/1			

Results and Discussion

COMPARISON OF MORPHOLOGY AMONG POPULATIONS

Due to the sexual dimorphism of *Luciobarbus* (Doadrio, 1990), and because few female specimens were available, we removed females from the subsequent morphological analyses. Non-parametric Kruskal–Wallis and Mann–Whitney *post hoc* comparison analyses were used to assess differences in size and body shape among populations (Table 1S). No differences in SL were found among *Luciobarbus* populations. However we found significant differences in morphometric measures, with the exception of preventral and preanal distances, anal and dorsal fin lengths, pre and post-orbital distances, and caudal peduncle length (Table 1S).

The population from Moulouya basin exhibited an elongated body shape with the smallest body depth of any studied population. The dorsal fin was placed slightly more anterior, and the predorsal distance was shorter, than in Tensift and Oum er Rbia populations. All fins in the Moulouya population were larger than the ones present in the remaining populations, with the height of the anal fin significantly bigger than in other populations, in some specimens reaching, or nearly reaching, the anterior rays of the caudal fin when folded. The population of Oum er Rbia Basin possessed the longest anal caudal peduncle. This population had a narrow skull, characterized by the lowest inter-orbital width. The population of Tensift Basin had the smallest head, with the head and prepectoral distances shorter than in Moulouya and Oum er Rbia populations. The deepest caudal peduncle was also found in the Tensift population. The fins in the Tensift population were significantly smaller than in Moulouya and Oum er Rbia populations. Some specimens of the Tensift population, belonging to paralectoypes of *Barbus magniatlantis*, presented three pairs of barbels, a characteristic that was not observed in any other populations. An overview of the general morphology of the analysed populations is illustrated in Figs 2 and 3.

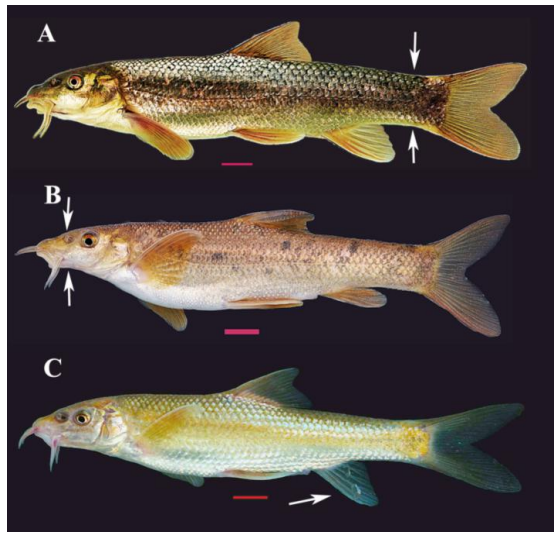

Fig. 2. Morphology of the analysed populations. Arrows show principal traits of each population. A: Tensift Basin population; B: Oum er Rbia Basin; C: Moulouya Basin. Scale: 10 mm.

Fig. 3.— Syntypes of *Barbus nasus* showing the characteristic deep caudal peduncle. Figure from the Natural History Museum (London).

The number of scales along the lateral line (median=45), superior transverse line (median=7.5) and inferior transverse line (median=5.5) was significantly lower in the Moulouya population than in the Tensift and Oum er Rbia (Table 2S). Scale number along the lateral line (median=51) and superior transverse line (median=9.5) were significantly higher in the Oum er Rbia population than in Tensift and Moulouya populations.

The principal component analysis (PCA) divided the specimens into three groups, corresponding to the populations of the three investigated

basins (Fig. 4). The eigenvalues of the three first principal components, with the Burnaby-corrected matrix, explained most of the variance (Table 2). The highest eigenvector values (anal fin height and inter-orbital width) were in agreement with results of Kruskal–Wallis and Mann–Whitney analyses (Table 1S).

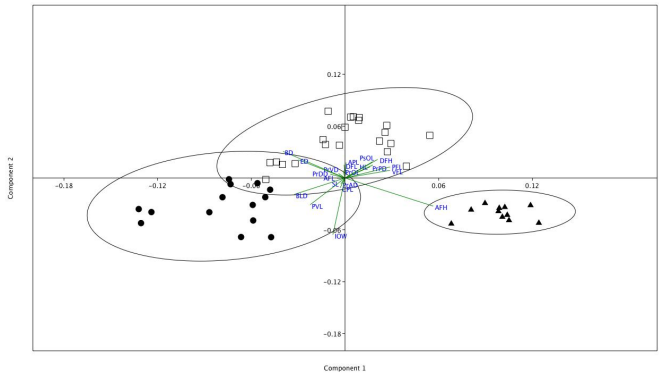

Fig. 4. Variables that most contributed to the PCA analysis. Dots: Tensift population. Squares: Oum er Rbia population. Triangle: Moulouya population. Acronyms are defined in Materials and Methods.

Table 2.— Eigenvalues and eigenvectors for the first three principal components (PC1-PC3) of 21 morphometric variables for all rheophilic *Luciobarbus* populations. Acronyms are defined in the Material and Methods section. Variables with the highest eigenvalues for each PC are in bold.

Variables	PCI	PCII	PCIII
Eigenvalue	0.0050	0.0019	0.0009
% variance	46.48	18.05	8.85
Eigenvectors			
SL	−0.2605	−0.0678	−0.1058
PrDD	−0.0926	0.0432	−0.1735
PrPD	0.1747	0.1477	0.0542
PrVD	−0.0406	0.0515	0.0110
PrAD	−0.0046	−0.0412	0.0021
PVL	−0.2190	**−0.3034**	**−0.3745**
CPL	−0.0301	−0.1112	**−0.3078**
APL	0.0062	0.1765	**−0.3697**
BD	**−0.3888**	**0.302**	0.1404
BLD	**−0.3187**	−0.1862	0.0311
HL	0.1575	0.1267	−0.1107
PrOL	−0.0114	0.0839	**0.4119**
ED	−0.2916	0.2065	0.1496
PsOL	0.1731	0.1844	−0.0512
PFL	0.2832	0.1257	0.1731
VFL	0.2832	0.0867	0.2835
DFL	−0.0053	0.1496	0.1400
DFH	0.2053	0.2113	−0.0097
AFL	−0.0504	−0.0029	−01324
AFH	**0.5536**	**−0.3194**	−0.1403
IOW	−0.0760	**−0.6432**	**0.4349**

OSTEOLOGICAL FEATURES

As shown by morphometric analyses, the skull of the Oum er Rbia population was narrower than the one present in Tensift and Moulouya populations. In particular, the ethmoid bone was longer and narrower (Fig.1S). In contrast, the kinethmoid bone of the Oum er Rbia population was shorter and more robust than in other populations (Fig.2S). The posterior branch of the lachrymal bone was elongated in the Oum er Rbia population and shorter in the Moulouya population (Fig.3S). The dentary bone was shorter in the Tensift population than in other populations (Fig. 4S). The anterior process of the maxilla of the Tensift population was lesser developed than in Oum Er Rbia and Moulouya populations (Fig.5S). The number of pharyngeal teeth in Tensift, and Oum er Rbia populations was usually 4.3.2. As in other *Luciobarbus* species, the juveniles possessed five teeth in the external row. In the Tensift population, the fifth tooth was occasionally retained in the adults. In the Moulouya population, we found pharyngeal teeth in a 4.2.1 or 4.3.1 configuration (Fig.6S). The Oum er Rbia population had a thick inner branch of the pharyngeal bone, with reduced pharyngeal lamina. In the Tensift population, the superior branch of the pharyngeal bone was strongly flexed (Fig.7.S).

In Oum er Rbia populations, the last single ray of the dorsal fin was strongly ossified with a maximum width of 12-14.5% (median=13.8) of its height (Fig. 5). In Tensift (6.7-9.5%, median=8.3) and Moulouya (6.2-9.2%, median=7.4) populations, the last single ray of the dorsal fin showed a lower level of ossification (Fig. 5). The Tensift and Moulouya populations showed weaker denticulations in the last single ray of the dorsal fin, but higher denticulate density (Moulouya 3.1-2.4 teeth/mm, median=2.6 teeth/mm; Tensift 2.2-1.8 teeth/mm, median=2.1 teeth/mm) (Fig. 5). The population from Oum er Rbia had scarce and strong denticulations on the last single ray of the dorsal fin at a density of 1.1-1.4 teeth/mm, median=1.2 teeth/mm

(Fig. 5).

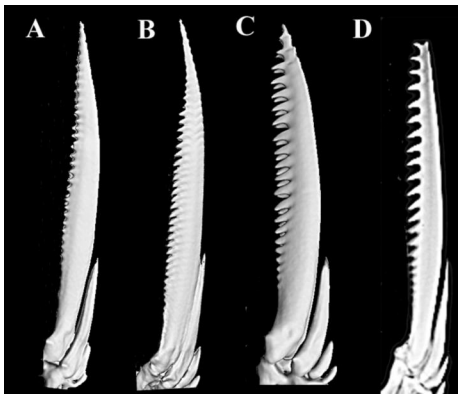

Fig. 5 Denticulation of the last single ray of the dorsal fin in specimens of the populations from Tensift (A, SL 135.8 mm), Moulouya (B, SL 139 mm), Oum er Rbia (C, SL 126.5 mm) and syntype of Barbus nasus (D, SL 108.7 mm).

MOLECULAR DATA

The phylogenetic analysis using BI, with *Aulopyge huegelli* and *Barbus meridionalis* as outgroup taxa, revealed two main clades corresponding primarily to the Iberian and African species (Fig. 6). The Iberian group clustered with *Luciobarbus setivimensis* of Algeria, as was previously reported (Machordom & Doadrio, 2001b).

Unexpectedly, the populations of rheophilic *Luciobarbus* were not monophyletic, and the Moulouya Basin population was clustered with the Iberian group, while the populations from Oum er Rbia and Tensift basins constituted a monophyletic group with *Luciobarbus ksibi* from the same basins and from its type locality (Kasab River). The phylogenetical position of the Moulouya population within the Iberian group was not resolved.

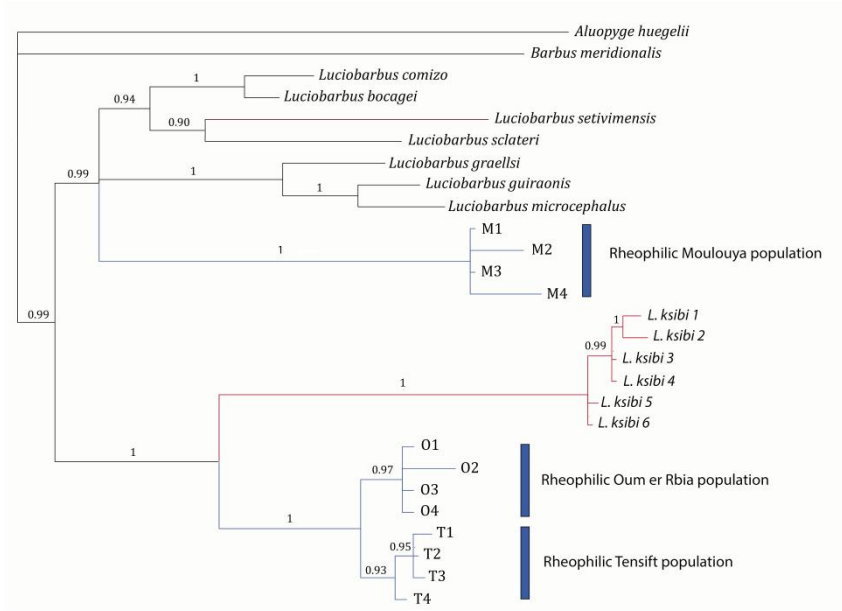

Fig. 6. Phylogenetic tree rendered by Bayesian Inference of the mitochondrial cytochrome b gene. Numbers on branches indicate posterior probability values. Identification of localities is defined in Table 1. Red branches show limnophilic North African species. Blue branches represent North African rheophilic populations.

Genetic distances within Moulouya, Oum er Rbia and Tensift populations of rheophilic barbels ranged from 0.1 to 0.3%. The Moulouya population showed genetic distances from Tensift and Oum er Rbia populations ranging from 8.1 to 8.4%. The genetic distances between Oum er Rbia and Tensift populations was 1.1% (Table 3). Genetic distances between limnophilic *L. ksibi* and rheophilic barbs of the same basins (Oum er Rbia and Tensift) were from 5.6 to 5.9%.

The genetic distances of Moulouya population with respect to other *Luciobarbus* species were similar or higher than the ones reported to sister species of cyprinid fishes (Doadrio & Carmona, 2003, 2006; Doadrio & Madeira, 2004; Robalo *et al.*, 2005; Doadrio & Elvira, 2007; Domínguez-Domínguez *et al.*, 2007, 2009). In contrast, genetic distances between Tensift and Oum er Rbia population were lower than the ones found in sister species of *Luciobarbus* (Casal-Lopez *et al.,* 2015).

Table 3.— Genetic distances for the complete mitochondrial cytb gene. Uncorrected-p genetic distances between species are below the diagonal. Uncorrected-p genetic distances within species are shown in the diagonal.

	Moulouya	**Oum er Rbia**	**Tensift**	***L. ksibi***
Moulouya	0.2			
Oum er Rbia	8.4	0.3		
Tensift	8.1	1.1	0.1	
L. ksibi	9.1	5.9	5.7	0.5

TAXONOMIC REMARKS

The three studied populations from the Oum er Rbia, Moulouya, and Tensift basins possessed molecular, osteological, and morphometric traits that identified them as different species. The assignment of Oum er Rbia, Moulouya, and Tensift populations to the rheophilic species *L. nasus* was not possible, as the combination of diagnostic traits of *L. nasus* was not shared by the studied populations. The combination of the diagnostic traits of all populations and the syntypes of *Barbus nasus* are showed in Table 4. The population morphologically most similar to *L. nasus* was that of the Oum er Rbia Basin. Differences between the Oum er Rbia population and *L. nasus* were found, primarily in the morphology of the last single ray of the dorsal fin, number of scales in the lateral line, morphology of the pharyngeal teeth, kinethmoid bone shape and caudal peduncle depth. The maximum width of the last single dorsal fin ray of the syntype of *Barbus nasus* was 11.5% of its height, and does not reach the robustness of that of the Oum er Rbia population (12-14.5%, median=13.8) (Fig. 5). The number of scales in the lateral line was 46-44 in *Barbus nasus* syntypes and 49-53 scales (x=51, median=51) in Oum er Rbia population. Pharyngeal teeth and kinethmoid bone were more robusts in Oum er Rbia than in *L. nasus* (Appendix 2-2, 2-7). Anal peduncle length was less of two times the BLD while in Oum er Rbia population was always more of two times.

In the absence of molecular data and a greater number of specimens for morphological studies, *L. nasus* must be considered an endemic species of the Kasab Basin and *L. magniatlantis* an endemic species of the Tensift Basin. The populations of Moulouya and Oum er Rbia basins exhibit multiple

diagnostic traits differing from those of *L. nasus* and *L. magniatlantis* (Table 2.S).

DESCRIPTION OF LUCIOBARBUS POPULATIONS

The high degree of morphological and genetic differentiation of *Luciobarbus* populations endemic to the Moulouya and Oum er Rbia basins justifies the consideration of these population as distinct species. No available names for these populations exist, and therefore these are described as new species in the present study.

Fig. 7.Holotype of *Luciobarbus guercifensis* from the Moulouya River, Ghafoula, Morocco. MNCN 290835. SL=139.4 mm.

Luciobarbus guercifensis sp. nov. Doadrio, Perea, & Yahyaoui

Holotype: Fig. 7, Table 4. MNCN 290835 male, 139.4 mm (SL); Moulouya River, Moulouya Basin, Ghafoula, Mediterranean slope in Morocco (34.14534, -3.38847) (Fig. 1); 22/6/2015. 378 ATSL, Collected by (Coll.) Doadrio, I; Yahyaoui, A and Perea, S.

Paratypes: Table 5. MNCN 286595-96: 2 specimens from Melloulou River, Moulouya Basin, Guercif, Mediterranean slope in Morocco (34.218035, -3.346732); 9/4/2007. Coll. Doadrio, I; Doadrio, I jr. and Perea, S. MNCN-290831: 1 specimen from Melloulou River, Moulouya Basin, Guercif, Mediterranean slope in Morocco (34.21526, -3.375668). 2/5/2015. Coll. Doadrio, I; Garzón, P; Perea, S and Yahyaoui, A. MNCN 290832: 1 specimen from Moulouya River, Moulouya Basin, Ghafoula, Mediterranean slope in Morocco (34.14534, -3.38847); 2/5/2015. Coll. Doadrio, I; Perea, S; Garzón, P and Yahyaoui, A. MNCN 290833-290834, 290836-290840: 7 specimens

from Moulouya River, Moulouya Basin, Ghafoula, Mediterranean slope in Morocco (34.14534, -3.38847); 22/6/2015. Coll. Doadrio, I; Perea, S and Yahyaoui, A.

The holotype and a series of paratypes (12 specimens) have been deposited at the Fish Collection of the Museo Nacional de Ciencias Naturales, Madrid, Spain.

Table 4. Morphometric and meristic measurement of the holotype and paratypes of *Luciobarbus guercifensis*. Acronyms are defined in the Material and Methods section.

	Holotype MNCN 290835	Paratypes n=11		
Morphometric variable	Measurements (mm)	Range	Mean	Standard Deviation
SL	139.4	75.7-149.4	109.4	23.5
PrDD	66.7	37.1-70.3	52.5	10.4
PrPD	35.2	20.1-38.2	28.3	5.7
PrVD	61.2	35.4-69.8	50.5	10.8
PrAD	97.7	49.7-107.8	75.9	18.4
PVL	30.4	15.4-34.3	24.3	5.6
CPL	58.1	30.9-60.6	44.6	9.5
APL	32.8	19.1-34.8	26.3	4.9
BD	26	12.4-28.1	19.5	4.9
BLD	14.9	7.8-16.2	11.5	2.7
HL	35	20.5-37.8	27.8	5.6
PrOL	17.3	8.1-18.8	12.6	3.2
ED	5	3-5.2	4	0.7
PsOL	15.4	8.7-16	12.1	2.3
IOW	10.5	5.1-11.3	7.9	1.9
B1L	9.7	4.7-11	7.4	2
B2L	12.8	7.1-13.7	10.2	2.1
PFL	30	16.8-33.3	24.1	4.9
VFL	27.3	16.8-31	22.7	4.5
DFL	17.1	9.2-17.7	13.4	2.8
DFH	27.2	15.7-28.5	22	4
AFL	11	5.8-12.1	8.6	2.0
AFH	26.4	16.3-28.5	22.1	3.9
CFL	32.5	16.5-34.1	26.1	5.3
LL	45	45-48	45.7	0.9
RSA	7.5	5.5-8.5	7.4	0.5
RSB	5.5	5.5	5.5	-

DIAGNOSIS: Differs from other known *Luciobarbus* species by the following combination of characters: 45-48 scales along the lateral line ($\bar{x}$=45.6 median=45), 6.5-8.5 ($\bar{x}$=7.4 median=7.5) above lateral line, and 5.5 below lateral line. The last single ray of the dorsal fin is strongly ossified with the maximum width 6.2-9.2 % (median=7.4) of its length. The last single dorsal

fin ray is densely denticulated along its length, and, in adults specimens, the number of denticulations exceeds 25 (Fig. 6). The ethmoid bone is wider than its length. Most specimens possess a single pharyngeal tooth in the inner row. The number of vertebrae is 39-41 ($\bar{x}$=39.7, n=11). The interorbital distance is 1.7 to 2.2 times (median=2) the eye diameter. The caudal peduncle is longer than the one seen in *L. nasus* and *L. magniatlantis*, with depth 2.1-2.6 times the length of the anal peduncle. The posterior segment (manubrium) of the lachrymal bone is short and high. Differences in diagnostic characters among analysed *Luciobarbus* populations are presented in Table 4.

DESCRIPTION: D III-V 8, A III 5, P I 15-16, V I 6, C 18; LL 45-48 ($\bar{x}$ =45.6, median=45), RSA 6.5-8.5 ($\bar{x}$ =7.4, median=7.5), RSB 5.5. Pharyngeal teeth in adults 4.3.1 or 4.2.1. GR 13-15 ($\bar{x}$ = 14.1, median=14), VE 39-41 ($\bar{x}$ = 39.7 median=40). Rarely reaches 200 mm SL. The body is elongated, relative to maximum body depth compared to other *Luciobarbus* species. Maximum body depth is 16-19% of SL. Head length ($\bar{x}$ = 28.5mm) is 25-27% of SL and greater than body depth ($\bar{x}$ = 20.1mm). The skull is wide, with the ethmoid bone width greater than its length. The interorbital distance is 1.7 to 2.2 times eye diameter. Infraorbital bones are narrow and the lachrymal bone is shorter than in Oum er Rbia population as consequence of a shorter manubrium. The height of the manubrium is 20-23% the lachrymal length. Thick lips and barbels display granular appearance. The first pair of barbels is short and located at the anterior extreme of the mouth, reaching the insertion of the second pair of barbels. The second pair of barbels is thick and reaches the posterior edge of the eye. The anterior barbel is 23.2-29.7%, and the second 34.6.38.6%, of HL. The snout is prominent, with preorbital length 10-12.6% of SL. The preorbital length ($\bar{x}$ =13.6mm) is larger than the postorbital length ($\bar{x}$ =12mm). The dorsal fin is posterior on the body but slightly more anterior than in other rheophilic barbs, with the predorsal distance being 46.2-50% of SL. The profile of the dorsal fin is concave, with the last single ray ossified with more than 25 denticulations (Fig. 6). In

males, the anal fin is longer than the one seen in *L. nasus* and *L. magniatlantis* with its height 76-92% of APL. The caudal peduncle is less deep than in *L. magniatlantis* and *L. nasus*, with a height 9.9-10.9% of SL. The length of the anal caudal peduncle is 2.1 to 2.6 times its height. In males, the pectoral and ventral fins are long with pectoral fins usually reaching the origin of the ventral fins. Males exhibited numerous nuptial tubercles of equal size distributed over the body and fins. The caudal fin length is 16.6-27.3% of SL. Morphometric and meristic measurements for the holotype and paratypes of *Luciobarbus guercifensis* are presented in Table 5. The colouration of *L. guercifensis* is silver-yellowish with a paler ventral area (Fig. 2).

DISTRIBUTION: This species is endemic to Moulouya basin, inhabiting Moulouya and Melloulou rivers in riffle areas near to the Guercif village (Fig. 1).

ETYMOLOGY: The species name 'guercifensis' was selected, as because the species is mainly distributed around the Guercif village in Morocco.

COMMON NAME: We propose the English common name 'Guercif barbel' for this new species.

HABITAT AND BIOLOGY: The species inhabits large rivers, mainly in mid-stream, usually in riffle areas. No information exists on the spawning period or reproductive behaviour.

CONSERVATION: Currently, *Luciobarbus guercifensis* is a rare species that has been found in few places, localized in riffle areas. These riffle areas are becoming scarce in Moulouya River as a consequence of increasing crop irrigation at its headwaters. The water taken for irrigation, as well as the impact of fertilizers and pesticides on water quality in the lower courses has likely been the primary cause of the recent decline of this population. No quantification of the decline in numbers is available. We suggest that this species should be included in the IUCN category of Endangered.

GENETICS: Uncorrected-*p* distance of mitochondrial cytb gene between

Luciobarbus guercifensis and the other analysed species are presented in Table 3. *L. guercifensis* shows 63 diagnostic positions in the cytb gene with respect to other rheophilic barbels.

Fig. 8. Holotype of *Luciobarbus zayanensis f*rom the Chbouka River, Oum er Rbia Basin. El Herri, Morocco. MNCN 279706. SL=113 mm.

Luciobarbus zayanensis sp. nov. Doadrio, Casal, & Yahyaoui

Holotype: Fig. 8, Table 5. MNCN 279706, male, 113 mm (SL); Chbouka River, Oum er Rbia Basin, El Herri (Elhri), Atlantic slope in Morocco (32.859510, -5.621355) (Fig. 1); 27/3/2013. 872 ATSL. Coll. Doadrio, I; Yahyaoui, A; Garzón, P and Perea, S.

Paratypes: Table 5. MNCN 55094 one specimen from the Douna River, Oum er Rbia Basin, El Khemis, Atlantic slope in Morocco (32.750866, -5.541695); 7/5/1988. Coll. Doadrio, I; Merino, M; Cubo J and González, JL. MNCN 208115-208116: 2 specimens from Serrou River, Oum er Rbia Basin, El Herri, Atlantic slope in Morocco (32.827621, -5.615255). 27/4/2000. Coll. Doadrio, I; Garzón, P; Doadrio, A and Doadrio, I Jr. MNCN 54987-55000: 14 specimens from the Arba River, Oum er Rbia Basin, Ksiba, Atlantic slope in Morocco (32.566810, -6.017450). 8/5/1988. Coll. Doadrio, I; Merino, M; Cubo J and González, J.L. MNCN 208168-208169, MNCN 71972-71978: 9 specimens from the Oum er Rbia River, El Borj, Atlantic slope in Morocco Morocco (33.015769, -5.629534). 18/4/1991. Coll. Doadrio, I; Perdices, A and Cubo, J. MNCN 279704-279705, MNCN 279707, MNCN 280083, MNCN 279695-279696: 6 specimens from the Chbouka River, Oum er Rbia Basin, El Herri, Atlantic slope in Morocco (32.859510, -5.62135527/3/2013. 27/3/2013. Coll. Doadrio, I; Yahyaoui, A; Garzón, P; and Perea, S.

Holotype and a series of paratypes (33 specimens) have been deposited at the Fish Collection of the Museo Nacional de Ciencias Naturales, Madrid, Spain.

Table 5. Morphometric and meristic measurement of the holotype and paratypes of *Luciobarbus zayanensis*. Acronyms are defined in the Material and Methods section.

	Holotype MNCN 279706	Paratypes n=32		
Morphometric measurement	Measurements (mm)	Range	Mean	Standard Deviation
SL	113	64.5-178.0	118.2	25.1
PrDD	56.7	33.3-92.5	59.5	12.7
PrPD	30	17.9-41.5	29.7	5.3
PrVD	54.7	33.3-79.4	55.1	10.5
PrAD	78.4	44.6-121.6	81.7	17.2
PVL	26.4	13.6-42.3	27.1	6.6
CPL	43.7	25.4-76.3	48.5	11.3
APL	28.3	16.0-45.2	29.8	6.3
BD	20.8	12.0-43.4	25.7	7.0
BLD	12.3	6.1-20.6	13.3	3.0
HL	28.9	17.4-43.4	29.6	5.8
PrOL	14.9	7.8-20.5	13.8	2.8
ED	4.6	3.5-6.3	4.7	0.6
PsOL	12	8.8-17.3	12.8	1.9
IOW	7.1	4.1-12.5	7.8	1.8
B1L	8.1	4.8-8.0	6.5	1.1
B2L	10.5	6.2-11.8	9.4	1.6
PFL	23.4	15.1-33.4	24.1	4.1
VFL	22.1	14.0-30.6	22.1	3.5
DFL	15.6	8.0-23.0	14.7	3.1
DFH	25	14.7-32.7	22.8	4.1
AFL	8.3	5.3-14.1	9.2	2.1
AFH	21	11.3-29.2	20.2	4.2
CFL	27.1	15.1-37.5	25.5	4.9
LL	51	49.0-53.0	51.1	1.2
RSA	9.5	8.5-9.5	9.3	0.4
RSB	5.5	5.5-7.5	6.6	0.6

DIAGNOSIS: Differs from other known species of *Luciobarbus* by the following combination of characters: 49-53 scales along the lateral line ($\bar{x}$ =51, median=51); 8.5-9.5 scales ($\bar{x}$ =9.3, median=9.5) above lateral line and 5.5-7.5 ($\bar{x}$ =6.5, median=6.5) below lateral line. The last single ray of the dorsal fin is strongly ossified with a maximum width of 12-14.5% (median=13.9%) of its length. The last single dorsal fin ray is densely

denticulate along its length and, in adults, the number of denticulations is smaller than 23 (Fig. 5). The skull is narrow, with the width of the ethmoid bone smaller than its length. The inter-orbital distance is 1.2 to 1.9 times (median=1.6) eye diameter. The pharyngeal teeth formula in adults is 4.3.2. The number of vertebrae is 41-43 ($\bar{x}$ =42, n=10). Large deep caudal peduncle 2.1-2.6% of APL. Lachrymal bone with narrow posterior segment (manubrium). Differences in diagnostic characters among analysed *Luciobarbus* populations are presented in Table 4.

DESCRIPTION: D III-V 8, A III 5, P I 15-16, V I 6, C 18; LL 49-53 ($\bar{x}$ =51, median=51), RSA 8.5-9.5 ($\bar{x}$ =9.3, median=9.5), RSB 5.5-7.5 ($\bar{x}$ =6.5, median=6.5). Pharyngeal teeth in adults 4.3.2. GR 13-16 ($\bar{x}$ =14.5 median=14.5), VE 41-43 ($\bar{x}$=42, n=10). Similarly to other rheophilic *Luciobarbus*, it is a small-sized species, rarely reaching 200 mm SL. Females are larger ($\bar{x}$ =128.6mm) than males ($\bar{x}$ = 112.9mm). The body is elongated with maximum body depth 18-24% of SL in males and 21-24% in females. The head is typically sharp and narrow. The inter-orbital distance is 1.2 to 1.9 times the eye diameter in males and 1.4 to 1.9 times in females. The skull is narrow, with the ethmoid bone longer than wide.

The frontal bones are narrower relative to other rheophilic barbs, notably in the anterior part. Infraorbital bones are narrow, and the lachrymal bone is longer than in other rheophilic barbs and possesses a long manubrium. The height of the manubrium is 10-13% the lachrymal bone length. The kinethmoid bone is more robust than the one found in other rheophilic barbs. Thick lips and barbels with granular appearance. The first pair of barbels is short and positioned at the anterior extreme of the mouth, reaching the insertion of the second pair of barbels. The second pair of barbels is thick and reaches the posterior edge of the eye. The anterior barbel length is 20.1-29.9%, and the second 29.7-39.5% of HL. The snout is prominent, with preorbital length 10.9-12.1% of SL in females and 10.6-13.2 in males. The preorbital length ($\bar{x}$ =14.8mm in females and $\bar{x}$ =13.5mm in males) is greater than the postorbital length ($\bar{x}$ =13.5mm in females and $\bar{x}$

=12.3mm in males). The pectoral and ventral fins are larger in males than in females. The pectoral fin length is 76-83% ($\bar{x}$=80%) of HL in females and 72-90% ($\bar{x}$=83%) of HL in males. The ventral fin length is 65.5-74% ($\bar{x}$ = 70%) of HL in females and 65.5-85% ($\bar{x}$=78%) of HL in males. The dorsal fin is located posteriorly on the body, with the predorsal distance being 48.9-52.5% of SL in females and 48.5-52.5% in males. The profile of the dorsal fin is concave, with the last single ray of the dorsal fin ossified with few and strong denticulations (Fig. 6). The caudal peduncle is elongated, and the least body depth is 11-11.9% SL in females and 9.5-11.8% SL in males. The length of the anal caudal peduncle is 2.1 to 2.3 times the height of the anal peduncle in females and 2.1-2.6 times in males. Males exhibited numerous equal-sized nuptial tubercles distributed over the body and fins. The caudal fin length is 20-23% of SL in females and 18.4-24.5% of SL in males. Morphometric and meristic measurements for the holotype and paratypes of *Luciobarbus zayanensis* are represented in Table 6. The coloration of *L. zayanensis* is brownish with a paler ventral area and some black spots along the body (Fig. 2).

DISTRIBUTION: This species is endemic to Oum er Rbia basin, inhabiting riffle areas with clear waters in the Atlas Mountains (Fig. 1).

ETYMOLOGY: The species name '*zayanensis*' has been selected because it is mainly distributed around Middle Atlas region, in the area inhabited by the Zayanes shepherds.

COMMON NAME: We suggest the English common name 'Zayan barbel' for this new species.

HABITAT AND BIOLOGY: The species inhabits riffle areas in headwaters of large rivers. Spawning takes places from late April to early May.

CONSERVATION: The habitat of *L. zayanensis* is being transformed by dam and reservoir constructions and by increased pollution linked to agriculture and urban development for tourism. The population of *L. zayanensis* is in decline, therefore we suggest that this species should be included in the IUCN category of Vulnerable.

GENETICS: Uncorrected-*p* distances of mitochondrial gene cytb between *L. zayanensis* and the other analysed species are presented in Table 3. *Luciobarbus zayanensis* shows 5 diagnostic positions in the cytb gene.

Supplementary material

Table 1S. Kruskal–Wallis test and Non-parametric Mann-Whitney's pairwise post hoc comparisons for all populations. Values of Kruskal-Wallis test (H) below variables. Values of Mann– Whitney test are below the diagonal. The median is the diagonal of each variable. Significant differences p<0.05 (*); p<0.01 (**). Acronyms are defined in the Material and Methods section.

Variables	**Populations**	**Tensift**	**Oum er Rbia**	**Moulouya**
		(n=14)	**(n=21)**	**(n=12)**
SL	Tensift	108.6		
(H=0.502)	Oum er Rbia	0.449	112.9	
	Moulouya	0.763	0.968	109.2
PrDD	Tensift	52.9/0.49		
(H=14.63**)	Oum er Rbia	0.88	55.8/0.5	
	Moulouya	<0.0001**	<0.0001**	53.3/0.48
PrPD	Tensift	25.2/0.24		
(H=16.54**)	Oum er Rbia	0.005**	29.4/0.26	
	Moulouya	<0.0001**	0.052	28.7/0.26
PrVD	Tensift	50.2/0.46		
(H=4.197)	Oum er Rbia	0.96	54.7/0.47	
	Moulouya	0.095	0.057	52/0.46
PrAD	Tensift	73.4/0.68		
(H=1.556)	Oum er Rbia	0.827	78.4/0.68	
	Moulouya	0.529	0.177	77.2/0.7
PVL	Tensift	24.9/0.24		
(H=13.73**)	Oum er Rbia	0.002**	24.9/0.22	
	Moulouya	0.0005**	0.8427	25/0.22
CPL	Tensift	42.7/0.41		
(H=1.89)	Oum er Rbia	0.372	44.2/0.4	
	Moulouya	0.978	0.177	44.3/0.41
APL	Tensift	25.3/0.24		

(H=17.8**)	Oum er Rbia	<0.0001**	28.3/0.25	
	Moulouya	0.019	0.06	26/0.24
BD	Tensift	22.4/0.21		
(H=24.63**)	Oum er Rbia	0.259	23.4/0.2	
	Moulouya	<0.0001**	<0.0001**	19.7/0.18
BLD	Tensift	12.8/0.12		
(H=34.48**)	Oum er Rbia	<0.0001**	12.5/0.11	
	Moulouya	<0.0001**	<0.0001**	11.7/0.11
HL	Tensift	25.1/0.24		
(H=29.07**	Oum er Rbia	<0.0001**	28.9/0.25	
	Moulouya	<0.0001**	0.234	28.5/0.25
PrOL	Tensift	12.7/0.12		
(H=2.33)	Oum er Rbia	0.578	13.5/0.12	
	Moulouya	0.106	0.365	13.6/0.11
PsOL	Tensift	11.1/0.1		
(H=22.8**)	Oum er Rbia	<0.0001**	12.3/0.11	
	Moulouya	0.0002**	8427	12/0.11
ED	Tensift	4.7/0.04		
(H=30.41**)	Oum er Rbia	0.002	4.6/0.04	
	Moulouya	<0.0001**	<0.0001**	4.2/0.04
PFL	Tensift	20.9/0.2		
(H=28.05**)	Oum er Rbia	0.0001**	24.2//0.21	
	Moulouya	<0.0001**	0.0009**	24.5/0.22
VFL	Tensift	19.4/0.18		
(H=24.74**)	Oum er Rbia	0.0009**	22.2/0.2	
	Moulouya	<0.0001**	0.001**	23.5/0.2
DFL	Tensift	13.8/0.12		
(H=3.7)	Oum er Rbia	0.699	14.8/0.13	
	Moulouya	0.132	0.074	13.6/0.12
DFH	Tensift	19.6/0.18		

(H=26,43**)	Oum er Rbia	<0.0001**	23.1/0.2	
	Moulouya	<0.0001**	0.7812	21.9/0.2
AFL	Tensift	8.3/0.08		
(H=1.27)	Oum er Rbia	0.88	9.1/0.08	
	Moulouya	0.218	0.451	8.9/0.08
AFH	Tensift	17.6/0.16		
(H=25.74**)	Oum er Rbia	0.1623	19.9/0.17	
	Moulouya	<0.0001**	<0.0001**	22.4/0.2
IOW	Tensift	7.9/0.07		
(H=32.72**)	Oum er Rbia	<0.0001**	7.1/0.06	
	Moulouya	0.132	<0.0001**	8.1/0.07
LL	Tensift	49		
(H=37.66**)	Oum er Rbia	<0.0001**	51	
	Moulouya	<0.0001**	<0.0001**	45
RSA	Tensift	8.5		
(H=40.5**)	Oum er Rbia	<0.0001**	9.5	
	Moulouya	<0.0001**	<0.0001**	7.5
RSB	Tensift	6.5		
(H=21.65**)	Oum er Rbia	0.03*	6.5	
	Moulouya	0.002	<0.0001**	5.5

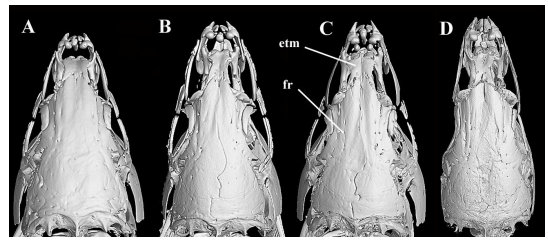

Fig.1S. Dorsal view of the skull of the populations under study. A: Tensift population (N'Fiss River), SL 135.8 mm. B: Oum er Rbia population (Chbouka river), SL 126.5 mm. C: Moulouya population (Moulouya River), SL 139 mm. D: Syntype of *Barbus nasus*, SL 108.7 mm. Etm = ethmoid bone, fr = frontal.

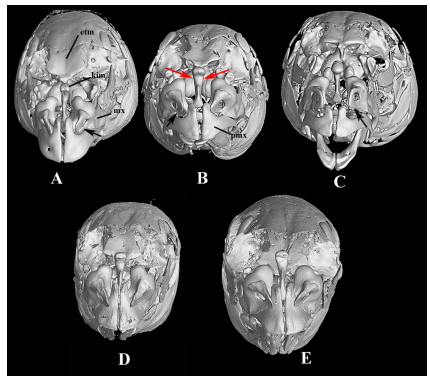

Fig.2.S Frontal view of the skull of the populations under study. A: Tensift population (N'Fiss River), SL 135.8 mm. B: Oum er Rbia population (Chbouka river) showed a short and wide kinethmoid bone, SL 126.5 mm. C: Moulouya population (Moulouya River), SL 139 mm. D: Syntype of *Barbus nasus*, SL 108.7 mm. E: Syntype of *Barbus nasus*, SL 81.5 mm. Etm = Ethmoid bone. Kim = Kinethmoid bone. Pmx = Premaxilla. Arrows show the width of kinethmoid bone.

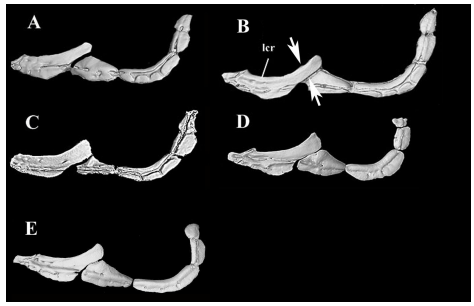

Fig.3.S Infraorbital bones of the studied populations. A: Tensift population (N'Fiss River), SL 135.8 mm. B: Oum er Rbia population (Chbouka river), SL 126.5 mm. C: Moulouya population (Moulouya River), SL 139 mm. D: Syntype of *Barbus nasus*, SL 108.7 mm. E: Syntype of *Barbus nasus*, SL 81.5 mm. Lcr = Lacrymal. Arrows show the width of lachrymal manubrium.

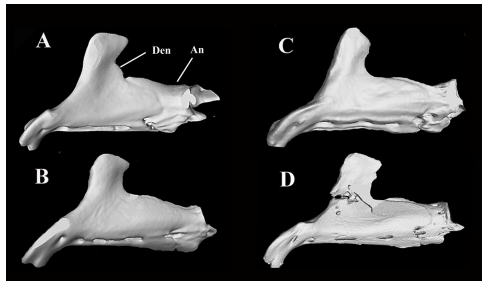

Fig.4.S. Dentary bone of the studied populations. A: Tensift population (N'Fiss River), SL 135.8 mm. B: Oum er Rbia population (Chbouka river), SL 126.5 mm. C: Moulouya population (Moulouya River), SL 139 mm D: Syntype of *Barbus nasus*, SL 108.7 mm. Den = Dentary. An = Anguloarticular.

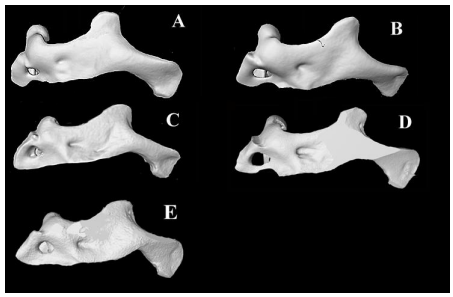

Fig.5S. Maxilla of the studied populations. A: Tensift population (N'Fiss River) showed a short anterior process, SL 135.8 mm. B: Oum er Rbia population (Chbouka River), SL 126.5 mm. C: Moulouya population (Moulouya River), SL 139 mm D: Syntype of Barbus nasus, SL 108.7 mm. E: Syntype of Barbus nasus, SL 81.5 mm.

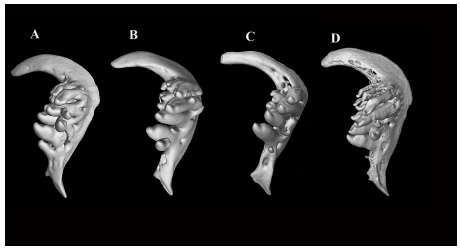

Fig.6S. Pharyngeal teeth of the populations studied. A: Tensift population (N'Fiss River) showed 5 teeth in the external row, 3 in the middle, and 2 in the inner row, SL 135.8. B: Oum er Rbia population (Chbouka river) showed 4 teeth in the external row, 3 in the middle, and 2 in the inner row, SL 126.5 mm. C: Moulouya population (Moulouya River) showed 4 teeth in the external row, 2 in the middle, and one in the inner row, SL 139 mm. D: Syntype of *Barbus nasus* showed 4.3.2 pharyngeal teeth, SL 81.5 mm.

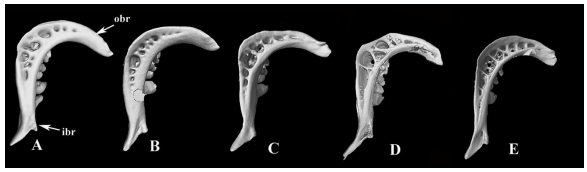

Fig.7S. External view of the pharyngeal teeth of the populations studied. Width of the inner branch between arrows. A: Tensift population (N'Fiss River) showed a flexed superior branch, SL 135.8 mm. B: Oum er Rbia population (Chbouka river) showed a strong inner branch, SL 126.5 mm. C: Moulouya population (Moulouya River) showed a thin pharyngeal bone. SL 139 mm. D: Syntype of *Barbus. nasus*, SL 108.7 mm. E: Syntype of *Barbus nasus*, SL 81.5 mm. ibr: Inner Branch. obr: Over branch.

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4. CHAPTER III

This chapter is based upon the manuscript:

Casal-López, M., Perea, S.,Sousa-Santos, C., Robalo, J.I., Torralva, M., Oliva-Paterna, F.J., Doadrio, I. (Under submission). Evaluating the influence of paleohidrology events on freshwater fish history through a model organism: the Iberian endemism *Luciobarbus sclateri* (Günther, 1868).

PALEOBIOGEOGRAPHY Of AN IBERIAN ENDEMISM, *LUCIOBARBUS SCLATERI* (GÜNTHER, 1868), INFERRED FROM MITOCHONDRIAL AND NUCLEAR MARKERS.

ABSTRACT

Since Cenozoic the Iberian Peninsula has undergone a series of complex geological and climatic changes, which have been considered as major forces acting on the evolutionary history of species from restricted areas. This complex past has shaped the freshwater network through time and the present-day distribution of primary freshwater species is expected to have been greatly influenced by these hydrogeological changes. Consider as a hotspot, the Iberian Peninsula is characterised by high levels of local endemicity. The species *Luciobarbus sclateri* (Günther, 1968) (Cyprinidae, Actinopterygii) is an endemism confined to southern Iberian Peninsula. Previous studies on *L. sclateri* drew a complex evolutionary history linked to scenarios of climate change during glaciations and left open the explanation of the genetic discordance found between the variation of the nuclear and the mitochondrial markers used. Based on this, the questions arising would be related to the populations structure of *L.sclateri* populations and to its biogeographical history, in origin and diversification. These questions have been constrained so far by a lack of a formal biogeographical methodology that could be applied to large number of areas, by the polyploid condition of the genus and by the limited number of populations included in previous studies, which did not cover the whole distribution range of the species. Our results suggest the isolation of the hydrographic basins as main factor driving intraspecific differentiation, followed by secondary contact, admixture and re-isolation.

Introduction

The Iberian Peninsula is located at the western area of the Mediterranean Basin and is characterized by a complex geological history due to its position between Eurasian and Africa land masses. Thus, Iberia has experienced several geological and climatic changes in its territory since the Cenozoic as a consequence of diverse events such as the uplifting of the Betic Mountains in the Serravalian and Tortonian periods (13.8-7.2 Ma), the Messinian Salinity Crisis (5.9-5.3 Ma), the Opening of the Strait of Gibraltar (5.3 Ma), and the Last Glacial Maximum (around 18 ka bp) (Hsü *et al.*, 1973,Rosenbaum *et al.*, 2001). The physiographical diversity of this area along with its geological history has shaped the evolutionary history of Iberian organisms making this region a suitable scenario for biogeographical studies (Buckley *et al.*, 1994,Buscak *et al.*, 2005,Perdices *et al.*, 1995,García-París & Jockusch, 1999, Machordom & Doadrio, 2011, Martínez-Solano *et al.*, 2006). Lots of these studies have focused on primary freshwater fish fauna division, which is comprised by species that are physiologically intolerant to marine conditions, dispersing via freshwater only which is restricted to river and lake drainage systems and show little capacity for transwatershed dispersal (Myers, 1938). Thus, their distribution is directly related to the paleogeographical evolution of the freshwater systems and the phylogenetic relationships and the population structure are expected to be closely linked to the history of hydrogeological network (Doadrio, 1990, Durand *et al.*, 1999, Sanjur *et al.* 2003, Reyjol *et al.*,2007). Barriers such as long-term basin boundaries are often considered more relevant to contemporary geographical patterns of freshwater fishes than current environmental conditions (Hugueny & Lévèque, 1994, Smith & Bermingham, 2005).

Iberian cyprinid species are primary freshwater fishes confined to specific ichthyogeographic provinces, inhabiting from one up to several river basins with almost no overlapping in their distribution ranges [Doadrio, 1998, Gante *et al.*, 2015). This mostly allopatric distribution pattern indicates that speciation in Iberian barbels follows the evolution of river basins (Doadrio *et al.*, 2002, ,Machordom &Doadrio, 2001, Mesquita *et al.*, 2007, Zardoya & Doadrio, 1998,1999). Secondary contacts in riverine species can be facilitated by several geomorphological processes affecting drainage patterns, such as river capture, marine regression and divide overtopping (Burridge *et al.*, 2008).

A typic element of primary freshwater fish fauna of the Iberian Peninsula is the genus *Luciobarbus* Heckel, 1843, which is widely distributed in Europe, North Africa and Asia (Doadrio, 1990, Zardoya & Doadrio, 1998, Doadrio *et al.*, 2002). In the Iberian Peninsula the genus *Luciobarbus* comprises six endemic species: *L. bocagei* (Steindachner, 1864), *L. comizo* (Steindachner, 1864), *L. graellsii* (Steindachner, 1866)*, L. guiraonis* (Steindachner, 1866)*, L. microcephalus* (Almaça, 1966) and *L. sclateri* (Günther, 1868).

In this study we focus on *Luciobarbus sclateri* (Güther, 1868), a polytypic generalized fish of the mid-south basins of the Iberian Peninsula including the Guadiana, Guadalquivir and Segura rivers basins (Doadrio *et al.*, 1991,Doadrio *et al.*, 2011).

Luciobarbus sclateri is a medium-large size species reaching 350 mm of standard length inhabiting middle and lower courses of Iberian southern rivers (Doadrio *et al.*, 2011). These rivers are characterized for Mediterranean regime with seasonal fluctuations (Oueslati *et al.*, 2015), which implies population fragmentation and depletion cyclically affecting the structure of these freshwater fish communities (Blondel & Aronson, 1999, Humphries & Baldwin, 2003, Magalhães *et al.*, 2002, Perea & Doadrio, 2015). These drainages, though representing a small hydrological area of

the Iberian Peninsula, contain considerable ichthyofaunistic value, with the presence of local endemic species, typically exhibiting a highly restricted distribution (Mesquita & Coelho, 2002).

Luciobarbus sclateri migrates upstream at the end of springtime for spawning during the flood period (Torralva *et al.*, 1997). As other primary freshwater fishes, *L. sclateri* is a non-salinity-tolerant species, so its active reproductive dispersion is limited to the interior of the basins where it is distributed (Doadrio & Perea, 2011). A limited dispersal propensity in organisms has been shown to facilitate the detection of phylogeographic patterns of population expansion by reducing the homogenizing effects of long-distance dispersal (Brito, 2005). Different studies have been carried out dealing with the species biology including, development, age, nutrition and gonad maturation (Castelló, 1981, Encina & Granado-Lorencio,1997, Lucena & Camacho, 1978, Oliva-Paterna *et al.*, 2003, Soriguer *et al.*,2000).

In the small basins of the southern Iberian Peninsula, *L. sclateri* is the most widely distributed species, and in some of them constitutes the unique species present (Doadrio *et al.*, 2011). This southern Iberian region comprises the Andalusian biogeographical sector (Doadrio, 1998), which has been currently split into four biogeographical provinces (Filipe et al, 2009). The Andalusian biogeographical area is particularly complex since it has undergone deep hydrogeological changes during the Upper Miocene and Lower Pliocene until reaching the present-day basins configuration in the Pliocene-Pleistocene, 2.5-1.8 Ma (Hsü *et al.*, 197, Calvo *et al.*, 1993, Andeweg *et al.* 1999).

A previous study based on mitochondrial markers and allozymes, indicated that *L. sclateri* population structure was determined by influence of the climatic oscillations during glaciations. As consequence different glacial refugia were found in southern Spain, an area previously considered to be scarcely affected by glaciations (Gante *et al.*, 2009). However, the explanation of the existence of discordance between the high level of

allozymic variation and the reduced level of mitochondrial variation was left open (Gante *et al.*, 2009).

Besides, studies dealing with the genetic diversity of *L. sclateri* in Portuguese basins, showed that the haplotype diversity was lower in comparison to other cyprinids but the highest diversity was found in Guadiana river, the largest one under study, than in the small ones (Sousa-Santos *et al.*, 2016). Previous genetic studies did not included most of the southern Spanish populations that comprise most of the distribution range of the species (Gante *et al.*, 2009). This fact, could have supposed a constraint in the reconstruction of the evolutionary history of *L. sclateri*, as it has happened in other phylogeographic studies in which the species range taken into account was limited (Avise, 2000, Barraclough & Nee, 2001, Hourlay *et al.*, 2008, King *et al.*, 1999, Kvist *et al.*, 2001, Marsh *et al.* 2004, Merilä *et al.*, 1997).

To unravel the patterns of diversification of *L. sclateri* and the origin of these patterns we used a mitochondrial (cytochrome *b* gene) and a nuclear intron (S7 gene), we carried out the most important sampling effort made so far including all the small southern basins around the Gibraltar Strait and we tested a formalized biogeographical analyses which has not been carried out till this study.

The aims of this study were the following: 1) Characterization of current genetic diversity within and among populations; 2) Estimation of the main cladogenetic events within the diversification process of the species; 3) Reconstruction of the demographical history of *L. sclateri* populations and 4) to infer the ancestral areas along the phylogeny in order to reconstruct the biogeographical patterns followed by this species throughout its diversification. 5) Finally, it was investigated whether the inferred processes are compatible with climatic oscillations in Late Pleistocene time frame.

Material and Methods

Sampling, DNA extraction, PCR and sequencing

The sampled area covered most of the known distribution range of *L. sclateri* (Table 1S, Fig. 1). Permission for sampling was obtained from the appropriate authorities. With the exception of a small number of specimens from each sampling site that were formalin preserved as voucher specimens, fin clips were taken, and fish were returned to the stream. Fin clips were preserved in 96% ethanol. Voucher tissues were deposited in the collections of the Museo Nacional de Ciencias Naturales (MNCN, Spain) and Instituto Universitário de Ciências Psicológicas, Sociais e da Vida (ISPA, Portugal).

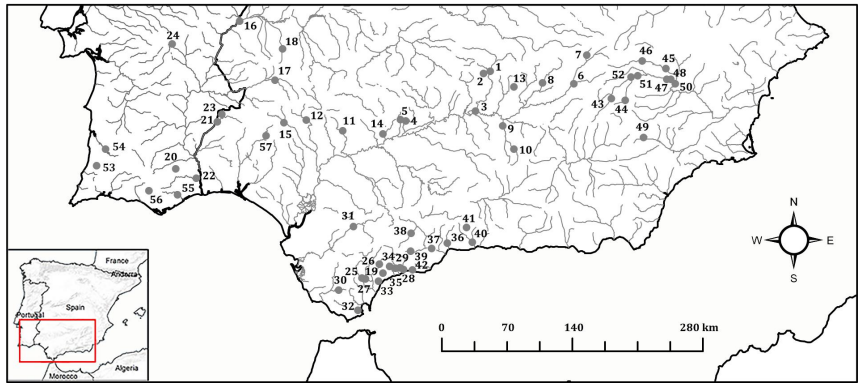


Fig.1.Map and Luciobarbus sclateri population sampling points: 1. Afluente río Montoro; 2. Robledillo; 3. Jándula; 4. Guadiato; 5. Cabrilla; 6. Guadalimar; 7. Guadalmena; 8. Guadalén; 9. Guadalbullón I; 10. Guadalbullón II; 11. Huéznar; 12. Cala; 13. Río de la Campana; 14. Bembézar; 15. Huelva; 16. Ardila; 17. De la Cueva; 18. Guadajira; 19. Padrón; 20. Vascao; 21. Chanza; 22. Odeleite; 23. Calaboza; 24.Degebe; 25. Hozgarganta; 26. Genal; 27. Guadiaro; 28. Verde; 29. Guadaiza; 30. Celemín; 31. Guadalete; 32. Miel; 33. Manilva; 34. Guadalmansa; 35. Guadalmina; 36. Guadalmedina; 37. Guadalhorce; 38. Turón; 39. Grande; 40. Posadas; 41. Guaro; 42. Real; 43. Zumeta; 44. Taibilla; 45. Mundo I; 46. Mundo II; 47. Segura I; 48. Segura II; 49. Caramel; 50. Mundo III; 51. Segura III; 52. Segura IV; 53. Seixe; 54. Mira; 55. Gilão; 56. Quarteira; 57. Odiel.

Total genomic DNA was extracted from fin tissue of each specimen, using the commercial kit Biosprint15 for tissue and blood (Qiagen). A fragment containing a nearly complete region (1140 bp) of the mitochondrial cytochrome b gene (*cytb*) and a fragment (475 bp) of the intron I of the S7 ribosomal protein gene paralog II (*S7II*) were amplified. Both genes have proved to be useful in resolving phylogenetic and phylogeographical relationships in cypriniforms. (Mayden *et al.*, 2009,Moyer *et al.*, 2009, Perea *et al.*, 2016, Schönhuth *et al.*, 2008, Sousa-Santos *et al.*, 2016). In the case of the genus *Luciobarbus* its tetraploid condition, due to recent genome duplication shows high sequence similarity between paralogs, especially protein-coding exons, which makes the use of nuclear genes a challenging issue (Gante *et al.* 2011). In our study we tested the two genes, S7 gene (paralog *S7I* and paralog *S7II*), and growth hormone (GH) gene (paralog *GHI* and paralog *GHII*), developed in (Gante *et al.*, 2011) for population and species level studies. Finally, we selected *S7II* based on the informative signal given. The *S7* gene has proven to be one of the most variable markers and it is been used in other freshwater fishes population studies (Alda *et al.*, 2013, Pérez-Rodríguez *et al.* 2009, Doadrio *et al.*, 2011). Therefore, even though the information obtained through the nuclear set was not as successful as expected, it was useful to shed light on some of the relationships found between populations.

Primers and protocols used for PCR for *cytb* and *S7II* are presented in Table 1. After checking PCR products on 1% agarose gels, they were purified by ExoSAP-IT ™ (USB, Cleveland, USA) and directly sequenced by MACROGEN Inc (Amsterdam, The Netherlands; http://www.macrogen.org) using a 3730XL DNA Sequencer.

Table 1. Primers and PCR condition

Gene	Primer name	Sequence 5'-3'	Tann (C^0)	Source
cytb	GludGL	TGACTTGAAR AACCA YCGTGG		(Palumbi,1996)
	H16460	CGAYC TTCGG ATTAA CAAGA CCG	48	(Doadrio & Perdices, 2001)
S7-II	Bs7-2.i1.269F	GGTGGCCCATATTTGCGGTA		
	Bs7-2.i1.785Ra	TGTCTGATTTATTAACTCCCAAA	54	(Gante *et al*, 2011)

Sequence alignment, Phylogenetic analysis and Haplotype network construction

All sequences were edited and aligned using Clustal W (Thompson *et al.*, 1994) as implemented in MEGA software v 5.1 (Tamura *et al.*, 2013). Chromatograms and alignments were revised and visually adjusted. Five hundred and five *L. sclateri* specimens from 22 basins and 53 locations were sequenced for *cytb*. Nuclear analysis (*S7II*) was conducted on a subset of ten samples per location when possible in order to try to diminish the effect of uneven sample size per location. From those, 211 individuals were successfully amplified. Those locations with or less than five individuals, came from small basins in the southern Iberian Peninsula with the exception of Mira Basin. Nonetheless, the effect of these small basins was balanced by pooling them together in the phylogroup-scale analyses.

We found in *S7II* PCR-amplified mixtures of two products that differ in length, corresponding to heterozygous individuals for several insertions or deletions (*indels*). This constraint the use of the software commonly used for phase data and thus, haplotypes were manually phased using the method described by (Sousa-Santos *et al.*, 2005, Flot *et al.*, 2006).

We computed the pairwise homoplasy index (Bruen *et al.*, 2006) for recombination using the software SplitTree v 4.10 (Huson & Bryant, 2006) to assess the presence or absence of genetic recombination in the nuclear *S7II* gene.

For phylogenetic analysis, identical sequences were collapsed using ALTER software (Glez-Peña *et al.*, 2010) in order to facilitate computational processes. The best-fit models of sequence evolution for the *cytb* and *S7II* under the Akaike Information Criterion (Akaike, 1973) were selected using JModeltest (Posada, 2008). The best model scheme found for the subsequent phylogenetic analyses was GTR+G (rate matrix: R(a)[AC] = 0.49; R(b)[AG] = 7.1; R(c)[AT] = 0.49, R(d)[CG] = 1.0; R(e)[CT] = 2.03; R(f)[GT] = 1.00; gamma shape = 0.166). For nuclear gene *S7II*, the best model scheme found was TPM3uf+G (rate matrix: R(a)[AC] = 4.55; R(b)[AG] = 8.6924; R(c)[AT] = 1.0, R(d)[CG] = 4.55; R(e)[CT] = 8.6924; R(f)[GT] = 1.00; gamma shape = 0.034). The nuclear gene *S7II* showed low polymorphism levels, and no structure was found in the phylogenetic analyses.

Bayesian inference was performed using MrBayes v 3.1.2 (Ronquist *et al.*, 2012). For the mitochondrial gene *cytb* Bayesian analyses were performed using two independent runs of four Markov Montecarlo coupled chains of 30^5 generations each to estimate the posterior probability distribution. Topologies were sampled every 1000 generations, and majority-rule consensus tree was estimated after discarding the first 10% of generations.

Phylogenetic analyses was conducted using Maximum Likelihood (ML) in RaxML (Stamatakis,2006,2014) software implemented in the Trex-online server (Boc & Makarenkov, 2012) using the substitution model GTRGAMMAI and the rapid bootstrap algorithm for c*ytb* analysis. Node confidence in analysis was estimated by rapid bootstrapping using a random seed (1000 replicates).

Based on previous phylogenetic studies (Levin *et al.*, 2012, Zardoya & Doadrio, 1999), *Luciobarbus callensis* and *Luciobarbus subquincunciatus* were used as outgroup in *cytb* Bayesian Inference tree and biogeographical reconstruction. *L. comizo* was used as outgroup for nuclear *S7II* phylogenetic inference based on the *cytb* analyses previously carried out in this study.

Haplotype networks for the *cytb* and *S7II* genes were obtained by using a median joining algorithm (Badnelt *et al.*, 1999) and edited in the software Network 4.5.1.6 (http://www.fluxus-engineering.com).

Estimation of divergence times

Divergence times for the *cytb* gene for *L. sclateri* populations and their credibility intervals were obtained with BEAST v 1.7.2 (Drummond *et al.*, 2012) using a lognormal relaxed clock, following a Birth-Death model of speciation and using fossil calibration. Markov Montecarlo chains were run for 50^6 generations with a sampling frequency of 10,000, and Tracer v 1.6 (Rambaut *et al.*, 2014) was used to assess convergence. The effective sample sizes for all parameters of interest were greater than 200. Finally, the trees were summarized with the software TreeAnnotator v.1.7.2 to obtain a maximum clade credibility tree with estimated divergence times (Drummond *et al.*, 2012).

To set the molecular clock, two calibration points were chosen based on fossil evidence for *Luciobarbus*: *Luciobarbus subtruncatus* (Heckel, 1843) from Vösendorf, Austria, dated ~10.5 Ma (Böhme, 2002) as the oldest known species within *Luciobarbus* and *Luciobarbus sp*. from the Iberian Peninsula dated ~6 Ma, considering the MEIN13 (Doadrio & Casado, 1989, García-Alix *et al.*, 2008, Sesé, 1989). Other species within the genus *Luciobarbus* were included in the analysis as shown in Table 2.S. Based on previous phylogenetic studies we selected *L. subquincunciatus* and *Capoeta*

sieboldii as outgroups to this most extended phylogeny (Levin *et al.*, 2012, Zardoya & Doadrio, 1999).

2.5. Genetic diversity, population structure and demography

Nucleotide and haplotype diversity parameters were estimated using DnaSP v 5.0 (Librado & Rozas, 2009). Genetic differentiation among populations was tested in Arlequin v 3.5.1.2. (Excoffier *et al.*, 2005) using the fixation index Φ_{ST} (Weir & Cockerham, 1984). Uncorrected *p*-distances among and within populations were also estimated for the *cytb* and *S7II* genes. Evidence for genetic hierarchical population structure was assessed by an analysis of molecular variance (AMOVA) for *cytb* and *S7II* genes, implemented in Arlequin v 3.5.1.2, with the aim of determining the genetic variability within and among populations. Overall differences in the entire dataset (one gene pool) were examined. A partitioning scheme of genetic variation based on phylogroups was also tested. To assess the influence of connections and barriers among sampled locations on the genetic structure of *L. sclateri* for *cytb*, a spatial analysis of molecular variance (SAMOVA) was conducted using the software SAMOVA 1.0 (Dupanloup *et al.*, 2002). SAMOVA was run from k = 1 to k = 20 with 100 simulated annealing processes and pair-wise differences to calculate the analogues of the fixation fixation indices (ϕ_{CT}, ϕ_{SC} and ϕ_{ST}).

To detect signatures of demographic shifts in the recent history of *L.sclateri*, deviations from a model of mutation-drift equilibrium were tested for the mitochondrial *cytb* gene, using Fu's F_s (Fu, 1997) with 1000 pseudo-replications. In addition, we carried out Tajima's *D* neutrality test (Tajima, 1989). The aforementioned tests were performed with DnaSP v 1.5 software (Librado & Rozas, 2009). The critical values obtained by simulations in neutrality tests were used to assess statistical significance ($p<0.02$ and $p < 0.05$, respectively) (Ramos-Onsins & Rozas, 2002).

To visualize historical demographic trends, we performed mismatch analyses in DnaSP v 5.0 (Librado & Rozas, 2009) for the *cytb* gene. We tested whether the data fitted the sudden demographic expansion model by assessing the probability of obtaining raggedness values (*Hri*) lower than observed (Rogers & Harpending, 1992) in a coalescent algorithm simulation over 1000 pseudo-replications with no recombination. We set initial values at θ_0 = 0 and θ_1 = 99,999. A Bayesian Skyline Plot (BSP) was created for several basins to reveal demographic changes over time as a measure of change in effective population size under neutral evolution (Drummond *et al.*, 2012).

Reconstruction of Ancestral Areas in *L. sclateri* phylogeny

We used a parametric approach in order to reconstruct the biogeography history of *L.sclateri* populations, due to its ability to integrate the estimates of the evolutionary divergence between lineages into the biogeographic inference (Sanmartin, 2012). We used the geological units corresponding to the Iberian basins as operational biogeographical units (OBUs) under study (Doadrio, 1998). Due to the large number of basins in the southern Iberian Peninsula and to their importance to the understanding of *L. sclateri* biogeography, the dispersal-extinction-cladogenesis model (DEC) described by (Ree *et al.*, 2005) could not be used due to its limitations in number area. Therefore, we used the BayArea method, a DEC-like approach, which has the advantage of numerically integrate all the possible cladogenetic events via Markov Chain Monte Carlo and it extends the application of biogeographical models to the analysis of situations involving a large number of areas (Landis *et al.*, 2013). BayArea method was implemented in software RASP 3.2 (Yu *et al.*, 2015). The results derived from this analysis should be carefully interpreted since under this model there are almost always fewer estimated vicariance than dispersal events.

BayArea calculates the ancestral states probabilities of each unit area with alternative burn-in values and presents the marginal probability for each alternative ancestral area as a pie chart at internal nodes (Tajima, 1989).

Basins may have become isolated, limiting dispersal and facilitating diversification of species. Therefore, the distribution range of *L. sclateri* was divided into the 22 basins where the species is currently present, coded as A, Guadalquivir; B, Guadiana; C, Odiel; D, Guadiaro; E, Guadalmina; F, Barbate; G, Guadalete; H, Miel; I, Manilva; J, Guadalmansa; K Verde; L, Guadalmedina; M, Guadalhorce; N, Vélez; O, Posadas; P, Real; Q, Padrón; R, Segura; S, Seixe; T, Mira; U, Gilao; V, Quarteira. We enforced a topological constraint corresponding to each node using the Bayesian tree, and the ancestral states for each node were reported. The analysis was conducted using a chain length of 50^6 sampled every 1000 generations and 100 trees randomly sampled from the posterior distribution of our Bayesian analyses after burn-in. Default values were used for the remaining parameters.

Results

Phylogeny, phylogeography, and molecular clock

The complete mitochondrial *cytb* gene (1140 pb) and a fragment of 475 bp for the nuclear *S7II* gene were sequenced from 505 individuals and 211 individuals, respectively. For the *cytb* gene, 95 characters were variable, of which 64 were parsimony informative and 31 were singletons. For *S7II*, 38 characters were variable, of which 24 were parsimony informative and 14 were singletons. For *cytb,* 76 haplotypes were obtained from the 505 sequences analysed. Pairwise genetic distances (uncorrected *p*-values) among populations ranged from 0.01% to 1.8% (Table 3S), with the highest genetic distances found between Barbate and Guadalmansa Basins. For *S7II*, 69 haplotypes were obtained from 422 sequences corresponding to the two phased alleles for each analysed individual. The maximum uncorrected

pairwise divergence between populations was 0.1% to 0.6% (Table 4S). The nuclear *S7II* gene showed 8 frequent heterozygous positions (18, 41, 72, 74, 198, 199, 238, and 330). Positions 198 and 199, corresponding to a change of bases from TC to AA, were found only in Guadalquivir Basin. Significant evidence for recombination was not found (p = 0.6052). For subsequent phylogenetic analyses, the *cytb* gene and *S7II* datasets were collapsed into 76 and 69 haplotypes/alleles, respectively.

Bayesian and Maximum Likelihood phylogenetic analyses for the *cytb* gene highly supported three main phylogroups among extant populations of *L. sclateri* (Fig. 2): phylogroup A, corresponding to a set of haplotypes unique to the Segura Basin; phylogroup B, comprising Real, Barbate, Miel, Guadiaro, and Manilva basins; and phylogroup C, including all the remaining populations. All basins included in phylogroup B, are small basins from the Mediterranean and Atlantic slopes of the Strait of Gibraltar. Phylogroup C encompasses the entire distribution area of *L. sclateri*, including basins in which haplotypes from phylogroups A and B are also present.

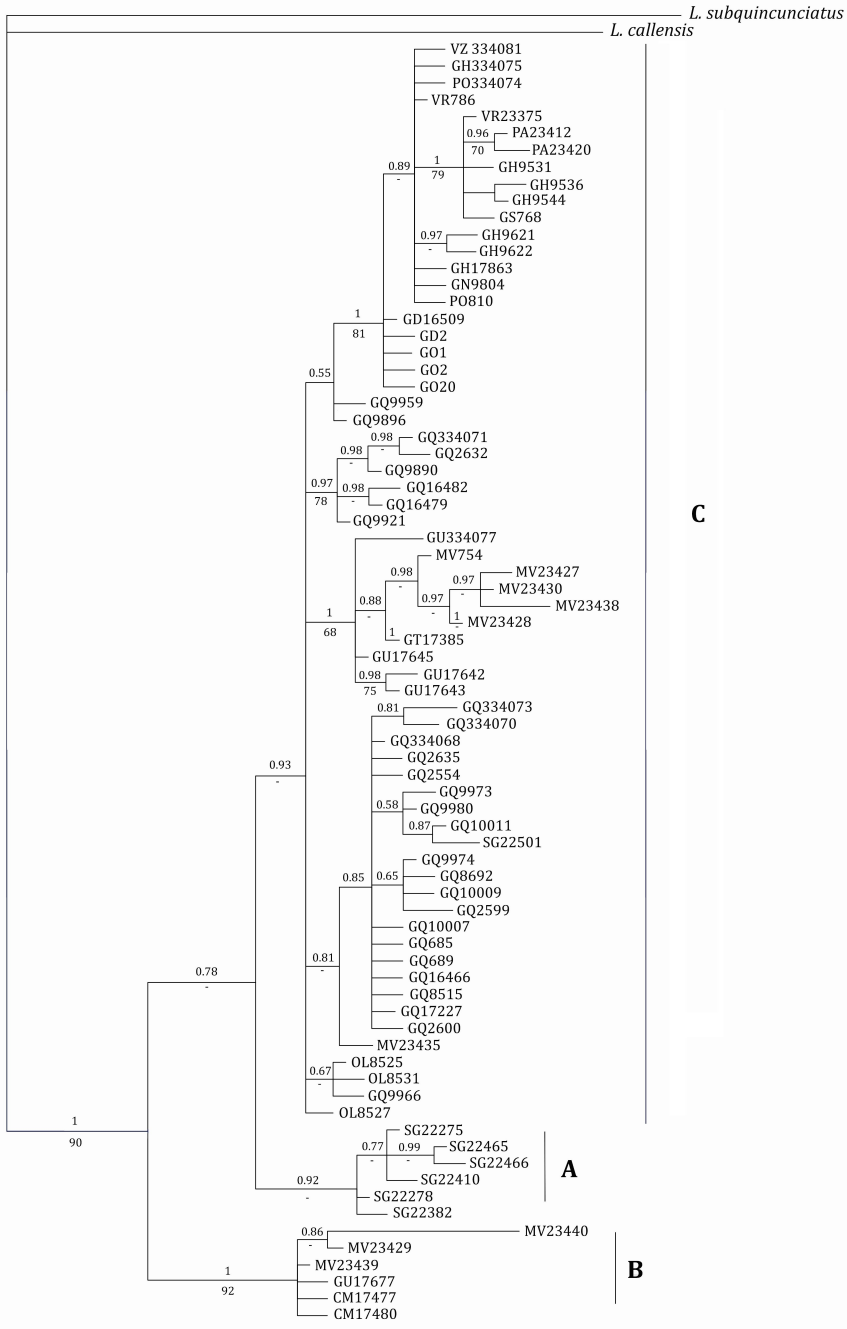


Fig.2. Phylogenetic tree of *cytb* gene rendered by Bayesian Inference (BI). Numbers on branches indicate posterior probability values for BI and under branches indicate bootstrap for ML.

The network analysis of the mitochondrial data also supports these three main phylogroups (Fig. 3). Phylogroup C shows remarkable haplotype richness and includes the Guadalquivir Basin, the basin containing the main population of *L. sclateri* (Doadrio *et al.*, 2011). Private haplotypes were found in Guadalquivir, Segura, Odiel, Guadalete, Guadalmedina, Guadalhorce, Padrón, Posadas, Gilão, and Manilva basins. The Guadiana river basin contains mainly shared haplotypes with the Portuguese basins, with the exception of Gilão, which also contains a unique haplotype.

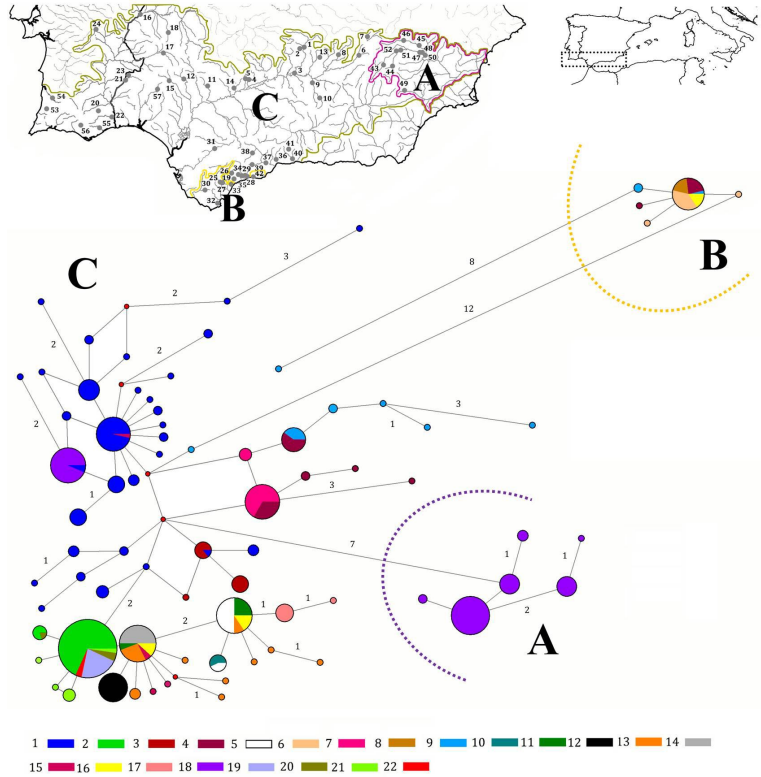


Fig.3. *Cytb* haplotype network. Mutations are indicated with numbers on branches linking haplotypes. Legend of colours shown corresponds to the code of the basin under study: 1. Guadalquivir (GQ); 2. Guadiana (GD); 3. Odiel (OL); 4. Guadiaro (GU); 5. Guadalmina(GM); 6. Barbate (CM); 7. Guadalete (GT); 8. Miel (ML); 9. Manilva (MV); 10. Guadalmansa (GS); 11. Verde (VR); 12. Guadalmedina (GN); 13. Guadalhorce (GH); 14. Vélez (VZ); 15. Posadas(PO); 16. Real (RE); 17.Padrón (PA); 18. Segura (SG); 19. Seixe (SX); 20. Mira (MI); 21. Gilão (GO); 22. Quarteira (QQ).

The nuclear phylogeny, based on the *S7II* gene, was not successful in solving relationships among groups, showing low support on the branches (Fig. 4), resulting in a polytomy, likely due to its low capacity for discriminating intraspecific differences between populations of *L. sclateri*. Nonetheless, the network analysis was able to recover, at the nuclear level, unique haplotypes such as those in the Guadalete, Gilão, Segura, Manilva, Guadiaro, and Guadalquivir basins, as well as several shared haplotypes reflected in the *cytb* haplotype network, including Guadiaro-Guadalete and Segura-Guadalquivir relationships. It is important to highlight that the Guadalquivir Basin showed a high nuclear diversity with a high number of private haplotypes. Curiously, Guadalquivir Basin is represented in a low frequency in the most common haplotype and also shared a common haplotype with Segura Basin (Figs. 3 and 4).

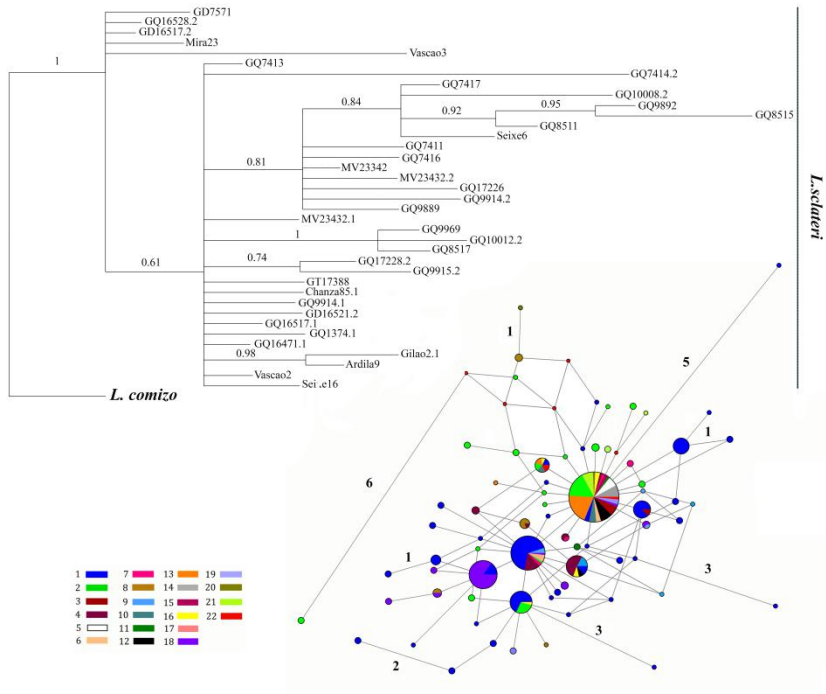


Fig 4.-S7II network and phylogram rendered by Bayesian Inference (BI). Mutations steps are indicated with numbers on branches linking alleles. Legend of colours shown corresponds to the code of the basin under study: 1. Guadalquivir (GQ); 2. Guadiana (GD); 3. Odiel (OL); 4. Guadiaro (GU); 5. Guadalmina(GM); 6. Barbate (CM); 7. Guadalete (GT); 8. Miel (ML); 9. Manilva (MV); 10. Guadalmansa (GS); 11. Verde (VR); 12. Guadalmedina (GN); 13. Guadalhorce (GH); 14. Vélez (VZ); 15. Posadas(PO); 16. Real (RE); 17.Padrón (PA); 18. Segura (SG); 19. Seixe (SX); 20. Mira (MI); 21. Gilão (GO); 22. Quarteira (QQ).

The split of *Luciobarbus sclateri* from other Iberian *Luciobarbus* species seems to have occurred during the Messinian Period (7.2-5.3 Ma). An initial event around 5.6 Ma separated *L. microcephalus*, *L. guiraonis*, and *L. graellsi* from the other Iberian species of *Luciobarbus* (Figure 5), which appear to have separated from its North African sister group *Luciobarbus setivimensis* around 4.88 Ma, corresponding to the Upper Miocene period, beginning the intraspecific diversification of *L. sclateri* around 3.17 Ma, in the Pliocene-Pleistocene period when the groups of basins corresponding to phylogroup B were the first to diverge, followed by phylogroup A, which began diverging 2.24 Ma. The remaining populations began diversification 1.9 Ma (Fig. 5).

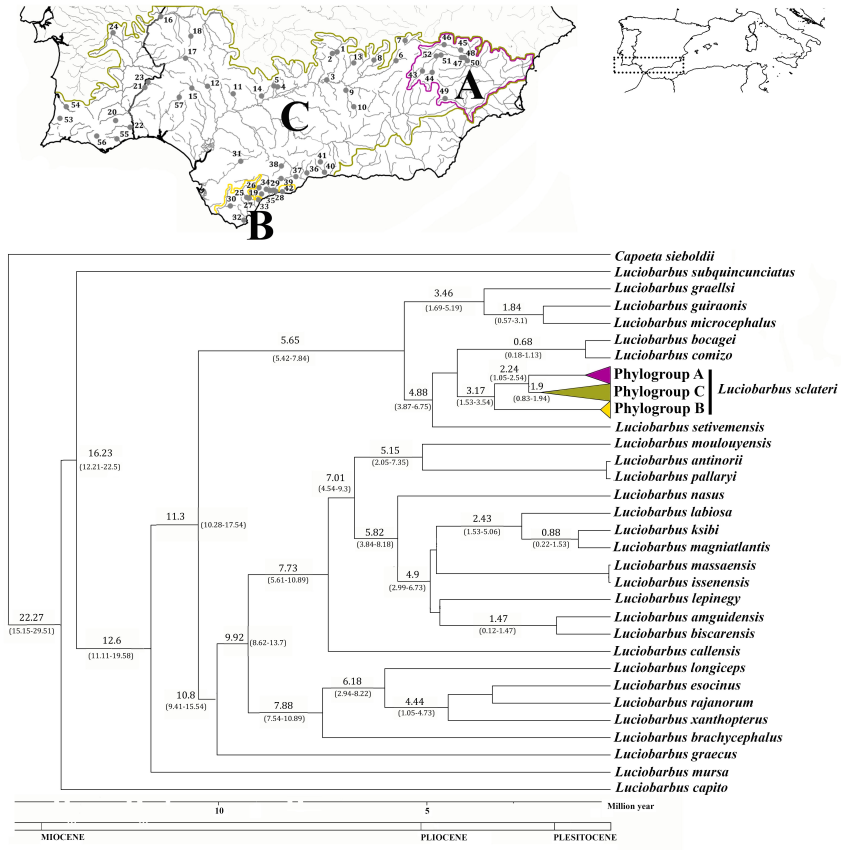


Fig 5.- Calibration tree and divergence times based on *cytb* gene and estimated through fossil record for the species comprising the genus *Luciobarbus* and the species of the genus *Capoeta* used as outgroups. Numbers, above and under branches, represent divergence ages estimation and their HPD 95% confidence intervals, respectively.

Genetic diversity, population structure, and population size

Overall genetic diversity for *cytb* showed a value of nucleotide diversity (π) of 0.0069, and haplotype diversity (*Hd*) of 0.941; with π of 0.004546 and *Hd* of 0.8701 for *S7II.* These parameters and others describing the genetic variability of aligned sequences are represented in Tables 2 and 3.

Based on the mitochondrial *cytb* gene the genetic diversity obtained higher values in two of the largest river basins, Guadalquivir (*Hd* 0.8908 and π 0.002559) and Segura (*Hd* 0.7274, π 0.004472), and in three small southern basins, Guadalhorce (*Hd* 0.8, π 0.0021), Manilva (*Hd* 0.858, π 0.0084), and Posadas (*Hd* 0.833, π 0.004). The lowest genetic diversity values were found in the following small southern basins: Vélez (*Hd* 0.118, π 0.0001), Padrón (*Hd* 0.222, π 0.000195), Barbate (*Hd* 0.3182, π 0.000292), and Guadalmina (*Hd* 0.281, π 0.000246). The diversity values of the Guadiana population were surprisingly low (*Hd* 0.1227, π 0.000108), considering that it is one of the largest basins in which *L. sclateri* occurs.

Table 2. *Cytb* diversity indices for whole data set and main basins (N= number of individuals analyzed; h= number of haplotypes; π= nucleotide diversity; H_D= haplotype diversity; S= number of polymorphic sites; SD= Standard deviations)

Basin	N	H	S	Hd(SD)	Π(SD)
Data Set	505	7	95	0.941(0.005)	0.00698(0.00022)
Guadalquivir	96	63 0	34	0.8908(0.0239)	0.002559(0.0015)
Guadalmina	19	2	1	0.281(0.116)	0.000246(0.000308)
Manilva	16	9	30	0.8583(0.0772)	0.008436(0.004573)
Verde	10	2	3	0.3556(0.1591)	0.000936(0.000761)
Padrón	9	2	1	0.222(0.1662)	0.000195(0.000287)
Segura	93	8	17	0.7274(0.0279)	0.004472(0.002425)
Guadiana	62	2	1	0.1227(0.0544)	0.000108(0.000188)
Guadalhorce	21	9	11	0.800(0.0776)	0.00216(0.001359)
Guadalmansa	4	1	0	0	0
Guadalete	25	3	4	0.3467(0.1078)	0.000503(0.000470)
Guadiaro	30	7	23	0.7770(0.0422)	0.0061(0.0032)
Real	13	3	20	0.7179(0.0571)	0.0082(0.004523)
Barbate	12	3	2	0.3182(0.1637)	0.000292(0.000352)
Miel	5	1	0	0	0
Gilão	8	4	2	0.7500(0.1391)	0.000815(0.000710)
Guadalmedina	21	1	0	0	0
Odiel	14	3	2	0.6044(0.0759)	0.000598(0.000543)
Mira	5	2	1	0.4(0.2373)	0.000351(0.000446)
Seixe	18	1	0	0	0
Quarteira	3	1	0	0	0
Posadas	4	3	3	0.833(0.2224)	0.0014(0.001274)
Vélez	17	2	1	0.118(0.101)	0.0001(0.00009)

As shown in Table 3, for the nuclear gene *S7II* the highest values of genetic diversity (*Hd* and π, respectively) were also obtained in Guadalquivir (0.8247 and 0.00549), Manilva (0.8485 and 0.0029), Guadiana (0.8183 and 0.005), Guadalete (0.8571 and 0.00274), and Posadas (0.833 and 0.003903) basins. The lowest diversity values were found in Gilão (0.2 and 0.000853) and Guadalhorce (0.197 and 0.000575) basins.

Table 3. *S7II* diversity indices for whole data set and main basins (N= number of alleles analyzed; h= number of alleles; π= nucleotide diversity; H_D= allele diversity; S= number of polymorphic sites; SD= Standard deviations)

Basin	N	H	S	Hd(SD)	Π(SD)
Data set	422	69	55	0.8701(0.0118)	0.004546(0.0027)
Guadalquivir	144	22	31	0.8247(0.0228)	0.00549(0.003275)
Guadalmina	10	2	2	0.356(0.159)	0.00152(0.0014)
Manilva	12	6	3	0.8485(0.0744)	0.002934(0.00218)
Verde	4	2	2	0.667(0.02041)	0.002837(0.002604)
Padrón	4	1	0	0	0
Segura	42	8	7	0.5203(0.0920)	0.002056(0.001586)
Guadiana	52	17	19	0.8183(0.0488)	0.005140(0.003144)
Guadalhorce	29	3	3	0.197(0.0952)	0.000575(0.000718)
Guadalmansa	6	1	0	0	0
Guadalete	8	4	3	0.8571(0.0818)	0.002741(0.002178)
Guadiaro	22	5	4	0.7143(0.0622)	0.002118(0.001657)
Real	4	2	2	0.667(0.2041)	0.002843(0.002610)
Barbate	8	2	1	0.5714(0.0945)	0.001218(0.001247)
Miel	6	2	4	0.5333(0.1721)	0.004539(0.00337)
Gilão	10	2	2	0.2(0.1541)	0.000853(0.000972)
Guadalmedina	8	1	0	0	0
Odiel	10	2	1	0.3556(0.1591)	0.000757(0.000903)
Mira	6	3	5	0.7333(0.1552)	0.005259(0.003802)
Seixe	8	3	4	0.7143(0.1227)	0.003655(0.002705)
Quarteira	2	1	0	0	0
Posadas	4	3	3	0.8333(0.224)	0.003903(0.003337)
Vélez	8	1	0	0	0

The AMOVA analyses of both *cytb* and *S7II* genes showed among-population ϕ_{CT} values higher for *cytb* than for the *S7II* if we consider one gene pool, 0.67 and 0.289, respectively (Tables 4 and 5).

Table 4. AMOVA genetic structure based on mitochondrial gene *cytb* of *Luciobarbus sclateri* populations among and within populations considering one gene pool.

Global diversity (One gene Group) *cytb*	df	Sum of square	Variance components	%Total	ϕ_{ST}	Significance tests(p-value)
			0.67550			**p<0.05**
Among Populations	21	1313.981	2.83659 Va	67.55		
Within populations	483	658.164	1.36266 Vb	32.45		
Total	504	1972.145	4.19925			

Within populations, the highest value was obtained for nuclear gene *S7II* at ϕ_{ST}= 0.71 compared to $\phi_{ST=}$0.541 for the *cytb* gene.

Table 5. AMOVA genetic structure based on nuclear gene S7II of *Luciobarbus sclateri* populations among and within populations considering one gene pool.

Global diversity (One gene Group) *S7*	df	Sum of square	Variance components	%Total	Φ_{CT}	Significance tests(p-value)
0.000			0.28948			
Among populations	21	133.119	0.32525 Va	28.95		
Within populations	402	320.921	0.79831 Vb	71.05		
Total	423	454.040	1.12356			

With SAMOVA, although p-values were significant (p-value<0.001) for all the K, the cluster K = 10 showed the highest ϕ_{CT} = 0.65. These ten groups are: group I: Guadiana and Portuguese basins, located at the southwestern of Iberian Peninsula; Group II, Odiel Basin; III group: Guadalquivir Basin and one tributary of Segura Basin; IV group, comprised by Guadalete, Guadiaro and Manilva basins; V, formed by Barbate and Miel basins, which are situated around the Strait of Gibraltar; VI, Verde, Guadalmina, Padrón, Guadalhorce, Real and Guadalmansa basins; VII group, comprised by Vélez, Guadalhorce, Posadas y Guadalmedina basins; VIII, Vélez Basin; IX and X, are comprised by different tributaries of Segura Basin.

This grouping has a geographic structure from west to east which is concordant with the current fluvial basins geographic situation with exception of Segura Basin, which is split into three different groups: two of them corresponding to Segura Basin itself, and the third one grouped with Guadalquivir (group III, IX and X). As for Guadalete Basin, which flows into the Atlantic slope, grouped with Mediterranean basins, Manilva and Guadiaro (group IV).

These relationships between no geographically-close basins, such as Guadalquivir-Segura, Guadiaro-Guadalete, have been already highlighted in the previous phylogenetic and network analyses. The geographical variable that sAMOVA takes into account, splits into more areas the three main phylogroups established by the previous phylogenetic analyses.

Based on mitochondrial DNA independent analysis of each basin, mismatch distributions showed unimodal graphics in all basins except for Guadalquivir, Segura, Guadiaro, Manilva, Real, and Guadalhorce. This suggests that some of them present population expansion. Nonetheless, when applying neutrality tests Fu's Fs, Tajima's D and R_2 for each independent basin, only Guadalquivir showed a significant negative value, suggesting an expansion of its population (Table 6). The non-significant Harpending raggedness index (*Hri)* corroborated the fit of the observed and expected distributions.

Table 6. *cytb* neutrality test and Harpening ragged index for each main basins: Fs (Fu's Fs test); D (Tajima's D test); R2 and Hri; p-values are show in brackets, significant values are highlighted in bold

Population: main Basins	Tajima's D(p) P<0.05	Fu's Fs(p) P<0.02	R2(p) P<0.05	Hri(p) P<0.05
Guadalquivir	**-1.73092(0.012)**	**-19.66207(0.000)**	**0.0325(0.008)**	**0.02108(0.68)**
Vélez	0.000 (p=1.000)	-	-	-
Guadalmina	-0.03486 (p>0.05)	0.42138(p>0.01)	0.1404(0.2899)	0.27116(0.25)
Manilva	0.26341(p>0.05)	1.12486(p>0.02)	0.1501(0.64)	0.02826(1.0)
Verde	0.02107(p>0.05)	2.33782(p>0.02)	0.1778(0.295)	0.668(0.36)
Padrón	-1.088823(p>0.05)	-0.26348(p>0.02)	0.3143(1)	0.358(0.31)
Guadiana	-0.53969(p>0.05)	-0.27610(p>0.02)	0.0366(0.069)	0.5845(0.32)
Segura	1.51442(p>0.94300)	5.80053(p>0.02)	0.1487(0.944)	0.1924(1)
Guadalhorce	-0.67663(p>0.05)	-2.31860(p>0.02)	0.0852(0.033)	0.1063(0.49)
Real	1.91710(p>0.05)	9.45997(p>0.02)	0.2335 (0)	0.321(0.02)
Guadalmansa	0.000 (p=1.000)	-	-	-
Guadalete	-1.23969(p>0.05)	0.21087(p>0.02)	0.1528(0.5337)	0.228(0.60)
Guadiaro	0.69047(p>0.05)	4.758 (p>0.02)	0.1458(0.806)	0.1295(0.32)
Celemín	-1.45138(p>0.05)	-1.32484(p>0.02)	0.1863(0.336)	0.2265(0.57)
Miel	0.000(p=1.000)	-	-	-
Gilão	0.75907(p>0.05)	-1.38724(p>0.02)	0.2416(0.6135)	0.2895(0.25)
Posadas	0.16667(p>0.05)	-0.13331(p>0.02)	0.2500(0.144)	1.4166(0.10)
Guadalmedina	0.000 (p=1.000)	-	-	-
Odiel	0.22615(p>0.05)	0.08624(p>0.02)	0.1863(0.522)	0.226(0.13)
Mira	-0.81650(p>0.05)	0.09021(p>0.02)	0.4000 (0.699)	0.2(0.97)
Seixe	0.000 (p=1.000)	-	-	-
Quarteira	0.000 (p=1.000)	-	-	-

Nucleotide diversity of *cytb* showed important differences among phylogroups (Table 7). The highest π was observed in phylogroup C, corresponding to Guadalquivir and the remaining basins within lineage C, with a π value of 0.004559 and Hd of 0.9227; followed by phylogroup A, which is the Segura cluster comprising unique haplotypes, with π of 0.000969 and Hd of 0.6109; to lineage B, formed by the Manilva, Real, Guadiaro, Miel, and Barbate basins, with π of 0.000812 and Hd of 0.3427.

Table 7. *cytb* diversity indices for phylogroups (N= number of samples analyzed; h= number of haplotypes; π= nucleotide diversity; H_D= haplotype diversity; S= number of polymorphic sites; SD= Standard deviations)

Phylogroup	**N**	**h**	**S**	**Hd(SD)**	**Π(SD)**
A	63	6	6	0.6109(0.0579)	0.000969(0.000713)
B	32	6	13	0.3427(0.1068)	0.000812(0.00064)
C	410	64	70	0.9227(0.0071)	0.004559(0.0024)

As shown in Table 8, AMOVA also supported the differences among the three main phylogroups, with variation among them of 60% (ϕ_{CT}=0.60001).

Table 8. AMOVA Genetic structure based on mitochondrial gene *cytb* of *Luciobarbus sclateri* phylogroups.

Global diversity(One gene Group) *cytb*	df	Sum of square	Variance components	%total	Significance tests(p-value) **p-value<0.05**
Among Populations	2	830.987	4.23361 Va	60.00	ϕ_{CT}=**0.60001**
Among populations within groups	23	859.261	2.06719 Vb	29.30	ϕ_{SC}=**0.73247**
Within populations	495	373.748	0.75505 Vc	10.70	$\phi_{ST:}$=**0.89299**
Total	520	2063.996	7.05585		

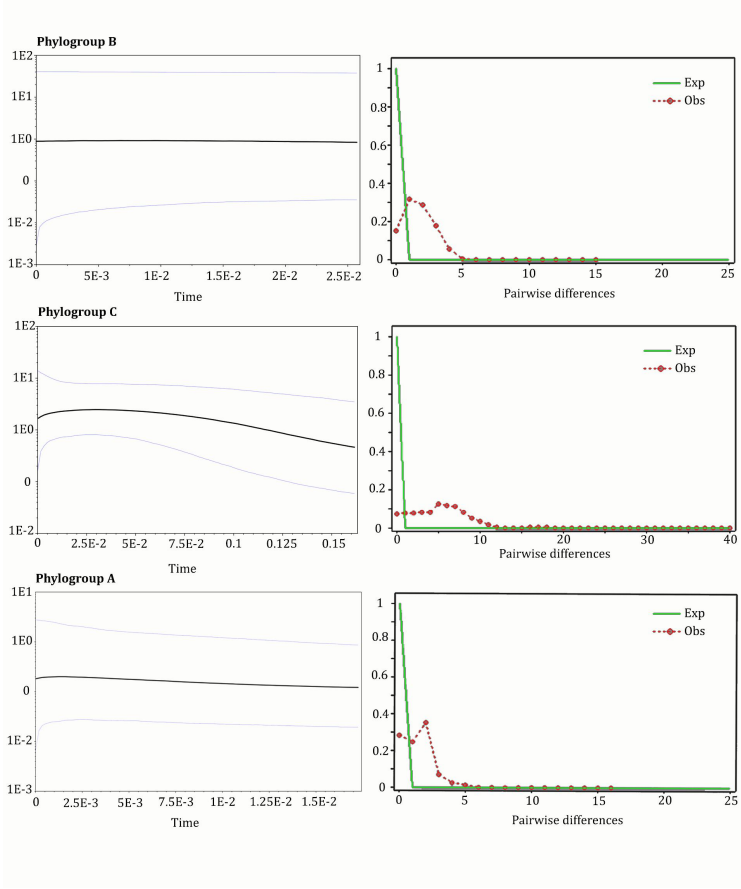
Table 9. *cytb* neutrality test and Harpening ragged index for each phylogroup: Fs (Fu's Fs test); D (Tajima's D test); R2 and Hri; p-values are show in brackets, significant values are highlighted in bold

Phylogroup	Tajima's D(p) p<0.05	Fu's Fs(p) P<0.02	R2(p) p<0.05	Hri(p) P<0.05
Phylogroup A	-0.3217(0.436)	-0.4961(0.404)	0.0918(0.415)	0.05423(0.85)
Phylogroup B	**-2.3244(0.003)**	-1.79809(0.09)	0.124 (0.43)	0.22054(0.59)
Phylogroup C	**-1.471(0.0310)**	**-24.774(0.001)**	**0.0325(0.032)**	**0.01537(0.53)**

Based on *cytb* data set, the Bayesian Skyline Plot analyses (BSP) (Fig. 6) of the three main phylogroups suggests stabilization of phylogroup B population to the present (Real, Barbate, Miel, Guadiaro, and Manilva Basins). The BSP of phylogroup C (Guadalquivir and the remaining basins within lineage C) showed population expansion extending from 150,000 years ago to 75,000 years ago, when the population reached a period of constant growth and recent decline. The BSP of Segura, which forms phylogroup A, indicated a consistent population maintained over time.

Nonetheless, mismatch curves for these phylogroups did not show unimodal distributions. For phylogroup C, neutrality tests, Hri and BSP did support a significant population expansion (Table 9 and Fig. 6).

Fig 6.- Bayesian Skyline Plots and mismatch distribution curves of phylogroups.



Biogeographical reconstruction

Ancestral area reconstruction using BayArea method (Fig. 7) showed a complex biogeographical history of the populations of *L. sclateri*, in which dispersal and vicariant events were frequent. In general, BayArea estimated 65 dispersal events, 10 vicariant events and 1 extinction event (in the ancestral area comprised by Guadalquivir, Odiel and Manilva basin) for the whole phylogenetic tree. The main nodes on the phylogenetic tree leading to the three main phylogroups are the following: Node 1 represents the split between clade B (comprised by the small southern basins around the Strait of Gibraltar) and the remaining populations. Node 2 represents the split of the populations of clade A (Segura basin), from the rest. Node 3 shows the split leading to the third main clade, C, comprising populations of Guadalquivir, Guadiaro, Guadalete, Odiel, Guadiana, and smaller southern basins.

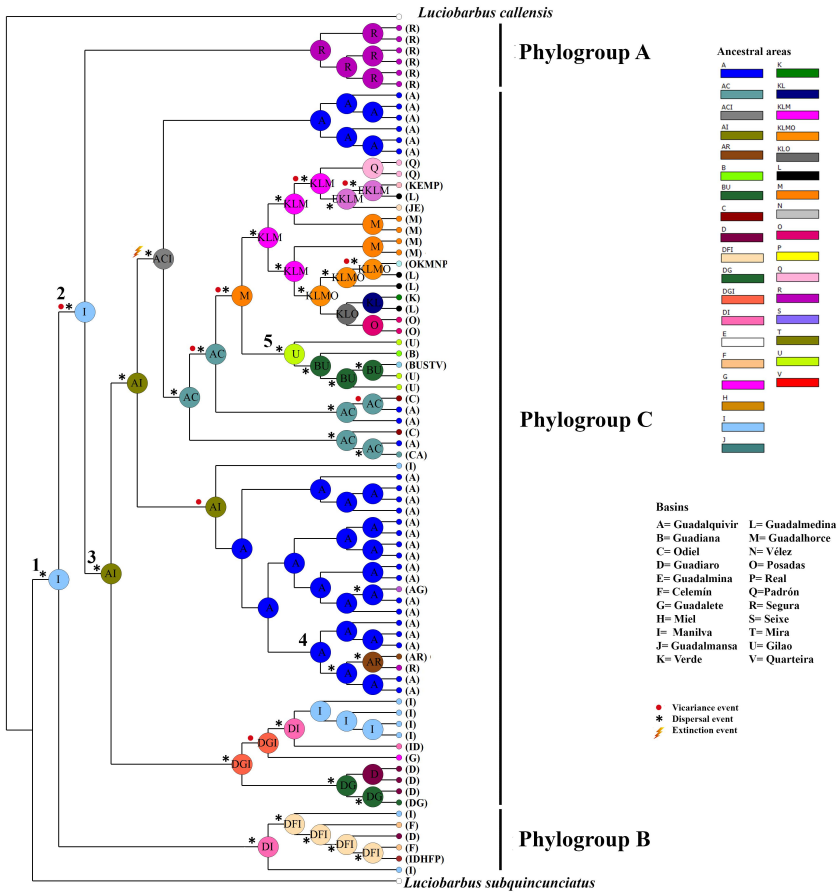


Fig. 7.- BayArea analysis of ancestral states of *Luciobarbus sclateri* population along the species distribution range on a tree generated by BEAST. Legend of colours represents possible ancestral ranges at different nodes generated by BayArea reconstruction. The Most Likely State is represented at the pie chart, with the code and colour corresponding to the area it is assigned. Biogeographical areas are comprised by 22 basins where the species is currently present, coded as: A, Guadalquivir; B, Guadiana; C, Odiel; D, Guadiaro; E, Guadalmina; F, Celemín; G, Guadalete; H, Miel; I, Manilva; J, Guadalmansa; K, Verde; L, Guadalmedina; M, Guadalhorce; N, Vélez; O, Posadas; P, Real; Q, Padrón; R, Segura; S, Seixe; T, Mira; U, Gilão; V, Quarteira.

Thus, from the ten vicariant events explained through BayArea (Fig. 7), the oldest vicariant event at node 1 splited most of the basins around the Strait of Gibraltar from the rest of the basins from southern Spain leading to clade B. Within the basins comprehending the area around the Strait of Gibraltar, the Manilva Basin corresponding to a karstic spring, was splited through a serie of vicariant events, leading to the current distribution of phylogroup B. An old vicariant event (node 2) divided the Mediterranean largest basin corresponding to Segura Basin (clade A) from the other southern basins. Another vicariant event involving the Segura Basin was the one separating individuals from Segura and northeastern tributaries from Guadalquivir Basin close to Segura from individuals of Guadalquivir Basin (node 4). An additional vicariant event that isolated Guadiana and small southern Portuguese basins should be highlighted (node 5). Sixty five different dispersal events were estimated through BayArea, making difficult to explain the geographical barriers that could have led to these vicariant events.

Discussion

This study has shown the imprint of paleohidrological processes in the population structure of *L. sclateri*, characterised by a complex history leading to present-day diverse watershed interconnections in southern Spain that have masked past relationships, in contrast to the clear patterns of population structure found in other freshwater fish populations inhabiting other areas of the Iberian Peninsula during the Plio-Pleistocene [Doadrio & Carmona, 2003, Lopes-Cunha *et al.*, 2012, Robalo *et al.*, 2007). This study has also shown that the lack of a complete sampling could highly affect the explanation of the evolutionary processes that have modelled the population structure and the phylogeographic models of *L. sclateri.*

A previous work highlighted that the main modulators of the evolutionary history of *L. sclateri* were the climatic oscillations occurring during the Pleistocene (Gante *et al.*, 2009). Nonetheless, a wider sampling of the populations of this species revealed that a pre-Pleistocene population structure exists, likely to have occurred during present-day basin formation, more linked to tectonic than to climatic changes. The presence of a great number of dispersal events during Pleistocene shown by our biogeographical analysis indicates a more complex history of inter-basin connection than in other areas of the Iberian Peninsula (Carmona *et al.*, 2000). These riverine connections are likely due to the location of southern Iberian in a geological stressed area placed between the European and African plates (Platt *et al.*, 2013, Santisteban & Schulte, 2007, Vergés & Fernández, 2012). Southern Spain is one of the most active seismic areas of the Iberian Peninsula and as a consequence, long-term fluvial network development has been significantly influenced by tectonics and river piracy (Santisteban & Schulte, 2007)

Our molecular calibration reveals that the first cladogenetic event within populations of *L. sclateri* was ~3 Ma, giving rise to the isolation of the southern basins around the Strait of Gibraltar (Phylogroup B; Fig. 5). This split is earlier than previously proposed (Gante *et al.* 2009), but is in agreement with the emersion of the basins located near the southwestern Iberian coastline, as Manilva, Vélez or Miel Basins during the Pliocene (Guerra-Merchán *et al.*, 2014). The Pliocene period also have been signaled to be an important period in the diversification of the freshwater fishes of Iberian Peninsula, such as *Squalius* and *Chondrostoma s.l.* (Almada & Sousa-Santos, 2010, Doadrio & Carmona, 2004, Durand *et al.*, 2003,Perea & Doadrio, 2015, Robalo *et al.*, 2007).

However, the occurrence of haplotypes in the populations of the southern basins distributed along the Strait of Gibraltar that are shared with other *L. sclateri* populations must be explained by dispersal events, as shown in BayArea analysis (Fig. 7).

These dispersal events have been favoured by the intense seismicity of the area as previously mentioned and also by the changes in the coastline, which have provoked basins-interconnectivity during the Pleistocene. Not only tectonic activity favoured dispersion but also the biology of the species itself did enable the rapid colonization of new basins when contacts occur through geographical metamorphosis, floods or confluence of river estuaries due to sea regressions associated with increased river flows (Bermingham & Martin, 1998). As other *Luciobarbus* species, *L. sclateri* performs long-distance migrations upstream to spawn in fresh and oxygenated waters (Banarescu & Bogutskaya, 2003) thus enhancing the probability of dispersion through secondary contacts among watersheds.

For these reasons, the population structure of *L. sclateri* is much more complex than those found in other small sedentary cyprinid fishes with the same distribution range, such as *Squalius malacitanus* and *S. pyrenaicus* (Perea *et al.* 2016. Sanjur *et al.* 2003). Therefore, the presence of shared Guadalquivir nuclear alleles and mitochondrial haplotypes with the small basins around the Gibraltar Strait seems to show a common history, reflecting recent contact (Figs. 3 and 4).

The second main cladogenetic event shown in the phylogenetic tree, network analyses of *cytb*, and BayArea analyses (Fig. 7) corresponds to isolation of Segura populations in the northeastern area of *L. sclateri* distribution (Phylogroup A). The present-day configuration of Segura Basin dates from Holocene but in the Late Pliocene important paleogeographical changes occurred in this area with a NNW-SSE rotation that changed the area orientation and provoked the formation of large sedimentary basins of freshwater origin in a zone previously occupied by ancient Betic paleomassifs (Montenat *et al.* 1990, Silva *et al.*, 1993, Silva, 2014).

The occurrence of a unique phylogroup corresponding to Segura basin seems to have originated due to the isolation of these basins during Pliocene and Holocene (Silva, 2014), reinforcing the idea that a tectonic cause could be claimed for the split of Segura phylogroup (Campuzano *et al.*, 1997). In addition to the unique haplotypes, several haplotypes and nuclear alleles that occur in the Segura Basin are also present in the Guadalquivir Basin (Figs. 3 and 4). These may be attributed to secondary contacts between the Guadalquivir and Segura Basins (Gante *et al.* 2009) mainly due to a piracy event from Segura to Guadalquivir basins. Retention of ancestral polymorphism is also a possibility, but the extensive divergence among the unique haplotypes of Segura and the ones shared with Guadalquivir Basin suggests secondary contact.

Squalius pyrenaicus is the only native cyprinid fish that currently occurs in the Segura Basin together to *Luciobarbus sclateri*. *S. pyrenaicus* is widely distributed in southern Spain, including the Guadalquivir Basin, but in the Mediterranean region, is present only in the Segura Basin and some single streams of the Ebro Basin (Doadrio *et al.*, 1991). Haplotypes found in previous studies for *cytb* of *Squalius pyrenaicus* also were clustered with Guadalquivir haplotypes, which reinforces the idea of a secondary contact between Guadalquivir and Segura (Doadrio *et al.* 2007).

The third clade (Phylogroup C Figs.2 and 3), including the rest of the basins and some of the genotypes from Segura and southern basins around the Gibraltar Strait, showed a dynamic history during the Pleistocene period as a consequence of frequent secondary contacts due to the interconnection of basins. Mitochondrial genes suggest the presence of a geographic pattern, although the analysis of the *S7II* sequences did not recover these relationships (Fig. 4).

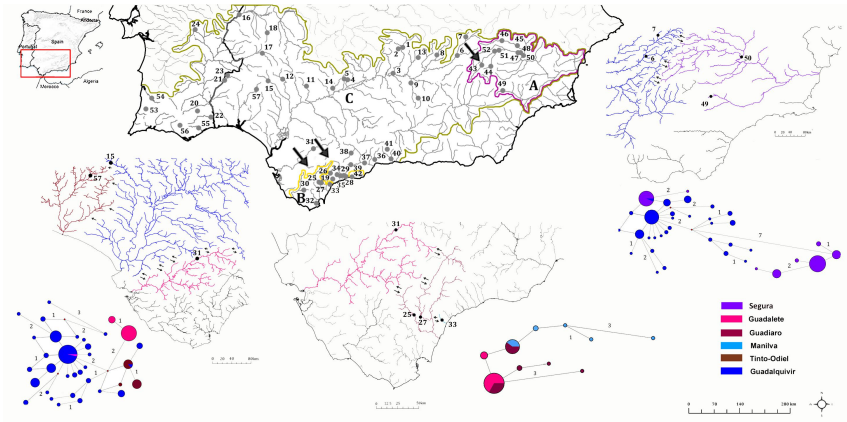


Fig 8.- Map highlightening the potential secondary contacts among the phylogroups A,B and C derived from results, along with the *cytb* networks related to the basins of interest.

Haplotypes of the Guadalquivir Basin populations were present in the adjacent basins of Tinto-Odiel and Guadalete (Figs. 3 and 8). In a broad scale, Guadalquivir, Guadalete, and Tinto-Odiel rivers shared a common geological history (Dabrio *et al.* 1998, Lario *et al.* 2002, Garcia & Lopez, 1984). At the end of Pliocene there was a primitive network that began to develop in a more complex fluvial network corresponding to the current rivers flowing into the Gulf of Cadiz (Rodriguez Ramírez *et al.* 1997, Rodriguez Vidal, 1989), which probably led to diverse fluvial contact between these basins. These secondary contacts could also be explained by a series of changes at the eustatic level during the Last Glacial Maximum when the coastline at Gibraltar Strait dropped 120 m, compared to current sea levels (Luque Ripoll *et al.* 1999, Dabrio *et al.* 1998, Garcia & López, 1984).

Relationships similar to those observed between Guadalete and Guadalquivir populations have been reported in other freshwater fishes (Gonzalez *et al.* 2014). It has been shown that in early Pleistocene time, Guadalete was tributary to Guadalquivir (Aguirre, 2004) and that the present-day configuration of Guadalquivir and Guadalete Basins involved several episodes of river captures (Rodriguez Vidal *et al.* 1993).

A similar history of secondary contacts due to dispersion was shown by BayArea analyses (Fig. 7) between Guadiaro Basin and its adjacent Manilva and Guadalete basins. Secondary contacts between Guadalete and Guadiaro populations could be possible since their headwaters are geographically close (Fig. 8).

Populations from the south-western basins in Portugal are clustered as were previously obtained by (Gante *et al.* 2009). The BayArea results explained this isolation by vicariance of this area with respect to the other southern basins, due to diverse geological events and the fault activity that took place during the Plio-Pleistocene, such as the formation of mountain chains such as Serra-do-Caldeirão, enclosing the Mira and Arade basins, during the early Pliocene or the Pleistocene-Holocene origin of Arade and

Quarteira basins (Ramos *et al.* 2015, Roque *et al.*, 2012, Chester *et al.*, 2012, Feio, 1952).

These dynamic processes with secondary contacts in a complex geographic area are reflected in demographic changes that may promote differences in genetic diversity. We expected drainage area size to reveal a positive correlation with genetic diversity indices, and this was the case, with some exceptions. The small basins of Manilva, Real, Gilão, and Posadas presented similar or higher genetic diversity values than the larger basins (Guadalquivir and Segura, respectively). This could be explained by recent secondary contacts between the small southern basins of Iberian Peninsula. In contrast, some large rivers, such as Guadiana and Guadalete, showed surprisingly low genetic diversity values in comparison with other large basins, as Guadalquivir Basin, which could be consequence of recent founder effects, as supported by the estimated divergence times. This lack of correlation between diversity indices and basin size has already been reported for other Iberian cyprinid fishes (Perea & Doadrio, 2015, Sousa-Santos *et al.* 2016).

When analyzing within-basin population size changes, we found agreement in neutrality tests (Tajima D, Fu's Fs, R_2), *Hri* index and BSP, showing significant expansion of the population of Guadalquivir Basin occurring around Würm II interphase period, which could have favored the expansion underwent by Guadalquivir population due to the warmer condition throughout this period (González-Sampériz *et al.* 2010, Carrión, 1992).

Conclusions

We hypothesized that a founder effect would have taken place, associated with migrations from the large basins to the smaller ones. Our reconstruction of the history of the populations of *Luciobarbus sclateri* reveals a series of vicariance and dispersal events that shaped its diversification in agreement with the complex geological changes occurred after of Messinian salinity crisis during Plio-Pleistocene. In general, basins within the distribution range of *L. sclateri* were characterized by shared haplotypes as well as private haplotypes. Therefore, our results show that the clustering of *L. sclateri* populations, which seems to be a reflection of diversification processes, is closely linked to the geological history of the hydrographic systems in which the species occurs. The mtDNA distribution patterns support the isolation of the hydrographic basins, which could be the main factor driving intraspecific differentiation, followed by secondary contacts, admixture, and re-isolation (Fig. 8). The initial hypothesis of a founder effect proceeding from large basins to smaller, followed by isolation associated with the separation of hydrographic basins is likely to have occurred on a recent temporal scale. Earlier geological events should be responsible for the isolation of southern basins during the Pliocene, and climatic factors are not necessary to explain the early divergence patterns shown by *L. sclateri*.

Supplementary material

Table 1S Sampling localities , molecular data codes and information for *Luciobarbus sclateri*. Uppercase letter between brackets refer to the basin code used.

Basin (code)	River	Map nº	Locality	*Cytb* haplotypes	*S7II* haplotypes
Guadalquivir (GQ)	~~Afluente río~~ Montoro	1	Solanilla del Tamaral, Ciudad Real	GQ334068,GQ2635,GQ2632	MV23342, MV23432.2
	Robledillo	2	Solana del Pino, Ciudad Real	GQ334068, GQ9921,GQ9890	GQ9969,GQ17228.2, GQ9914.1,GQ9914.2,GQ9915.2 ,GQ7413
	Jándula	3	Andújar. Jaén	GQ334068,GQ17227,OL8525	GQ7417, GQ7411, GQ17228.2, GQ17226
	Guadiato	4	Santa María de Trasierra, Córdoba	GQ2599, GQ2600,GQ9974, GQ17227	GQ7413, MV23342
	Cabrilla	5	Villaviciosa de Córdoba, Córdoba	GQ8692	MV23342
	Guadalimar	6	Arroyo del Ojauco. Jaén	GQ9973,GQ9974, GQ9980, GQ334068	MV23342, GQ7417
	Guadalmena	7	Albaladejo, Ciudad Real	GQ9973, GQ9980, GQ10011	MV23342, GQ9969, GQ10008, GQ10012.2, GQ7413, GQ7417
	Guadalén	8	Aldeaquema. Jaén	GQ9959, GQ334068, GQ9966	MV23342, GQ9969, GQ7417
	Guadalbullón I	9	Las Infantas. Jaén	GQ685	GQ9969
	Guadalbullon II	10	La Cerradura. Jaén	GQ689	GQ9969
	Huéznar	11	Constantina, Sevilla	GQ334068, GQ8515	MV23342, GQ8511, GQ8511.2, GQ8515, GQ8515.2, GQ8517
	Cala	12	Santa Olla de Cala. Huelva	GT17385, GQ16479	GQ7413, GQ7414.2, GQ7417, GQ7411, GQ7416
	De la Campana	13	La Carolina, Jaén	GQ334071, GQ334068, GQ9896	GQ9892, GQ9889
	Bembézar	14	Hornachuelas. Córdoba	GQ334068,	GQ7413, GQ7417, GQ10012.2
	Huelva	15	Cortelazor, Huelva	GQ334068,GQ9974	GQ16471.1
Guadiana (GD)	Ardila	16	Valverde de ~~Burdillos~~, Badajoz	GD16509	GQ7413, GQ7417, Ardila 9
	Calaboza	23	Rosal de la Frontera, Huelva	GD16509	GQ7413, GQ7417, MV23342, GD16528, GD16521, GD16517.1, GD16517.2
	Guadajira	18	Villalba de los Barros, Badajoz	GD16509	GD7571
	Chanza	21		GD16509, GD2	Chanza85
	Vascao	20		GD16509	Vascao3, Vascao2
	Odeleite	22		GD16509, GD2	GQ7413, GQ7417
	Degebe	24		GD16509	GD16528.2
Odiel (OL)	Odiel	57	Campofrío, Huelva	OL8525, OL8527, OL8531	GQ7413
Guadiaro(GU)	Guadiaro	27	Jimena de la Frontera, Cádiz	GU334077, MV754, MV23439, GU17645,	MV23342, MV23432.2, GQ7413
	Genal	26	Jubrique, Málaga	GU17642, GU17643,	
	Hozgarganta	25	Jimena de la Frontera, Cádiz	GU17677	
Guadalmina (GM)	Guadalmina	35	Benahavis, Málaga	VR23375, GS768	GQ7413, MV23432.2
Barbate (CM)	Celemín	30	Benalup-Casas Viejas, Cádiz	CM17477, CM17480, MV23439	GQ7413, MV23342
Guadalete (GT)	Guadalete	31	Puerto Serrano, Cádiz	GU17645, GT17385, GQ334068,	GT17388, GQ7413, MV23342
Miel (ML)	Miel	32	Algeciras, Cádiz	MV23439	MV23342, MV23432.2
Manilva (MV)	Manilva	33	Manilva, Málaga	MV23440, MV754, MV23427, MV23428, MV23429, MV23430, MV23435, MV23438, MV23439, MV23440	MV23342, MV23432.1, MV23432.2 , GQ7413
Guadalmansa(GS)	Guadalmansa	34	Benahavis, Málaga	GS768	GQ7413
Verde (VR)	Verde	28	Istán, Málaga	VZ334081, VR23375, VR786	GQ7413, MV23342
Guadalmedina (GN)	Guadalmedina	36	Málaga	GN9804	GQ7413
Guadalhorce (GH)	Guadalhorce	37	Cártama,Málaga	VZ334081, GH334075, VR23375	GQ7413

	Turón	38	~~Ronda,~~ Málaga	GH9531,GH9536,GH9544, GH9621,GH9622,GH17863	
	Grande	39	Ronda. Málaga		
Vélez (VZ)	De la Cueva	17	Riogordo, Málaga	VZ334081	GQ7413
	Guaro	41	Portugalejo, Málaga		
Posadas (PO)	De la Posadas	40	Mijas, Málaga	VZ334081, PO334074, PO810	GQ7413
Real (RE)	Real	42	Marbella, Málaga	VZ334081, VR23374, MV23439	GQ7413, GQ7417, MV23432.2
Padron (PA)	Padrón	19	Estepona, Málaga	PA23412, PA23420	GQ7413
Segura (SG)	Mundo I	45		GQ10011, SG22275, SG22278, SG22465,	GQ7413, MV23342, MV23432.1
	Mundo II	46		SG22410, SG22382, SG22501, SG22466	
	Mundo III	50			
	Segura I	47			
	Segura II	48			
	Segura III	51			
	Segura IV	52			
	Caramel	49			
	Taibilla	44			
	Zumeta	43			
Seixe (SX)		53		GD16509	
Mira (MI)		54		GD16509, GD2	
Gilão (GO)		55		GO1, GO2, GO20	
Quarteira (QQ)		56		GD16509	

Table 3S. Outgroups and species within the genus *Luciobarbus* included in phylogenetic analysis and molecular clock.

Species	**River, Location**	**Accession nos.**
Capoeta sieboldi	Kizilirmak River, Black Sea Basin, Turkey	JF98329
Capoeta caelestis	Goksu River, Turkey	JF798286
Barbus haasi	Ebro, Mesa, Jaraba, Spain	AF334101
Luciobarbus subquincunciatus	Kebam dam lake, Euphrates river Basin, Turkey	AF145937
Luciobarbus graellsi	Tiber River, Central Italy	JN049525
Luciobarbus guiraonis	Buyent River, Buyent Basin, Spain	AF045972
Luciobarbus microcephalus	Guadiana River , Zujar, Peraleda de Zaucejo, Spain	AF334085
Luciobarbus bocagei	Tajo, Uso, Aldeanueva de San Bartolome, Spain	AF334053
Luciobarbus comizo	Guadiana, Albuera, Almendral, Spain	AF334050
Luciobarbus setivemensis	Soumman, Takretz, Algeria	AY004748
Luciobarbus ~~moulouyensis~~	Moulouya, Boumia, Morocco	AY004742
Luciobarbus antinorii	Bichri, Fatnassa, Tunisia	AY004725
Luciobarbus payarii	Ain Dzadert, Figuig, Morocco	AY004736
Luciobarbus nasus	Oum Er Rbia R., Morocco	AF145924
Luciobarbus labiosa	Soltane R, Tattofte, Morocco	JF798259
Luciobarbus ksibi	Kasab, Essaouira, Morocco	AY004738
Luciobarbus magniatlantis	Oum Er Rbia, El Borj, Morocco	AY004734
Luciobarbus massaensis	Zag Mouzen, Tiliouine, Morocco	AY004737
Luciobarbus issenensis	Souss R., Morocco	AF145928
Luciobarbus callensis	Kebir River, Algeria	AF045974
Luciobarbus lepineyii	Dra River, Morocco	JF798261
Luciobarbus amguidensis	Imirhou Riverl, Algeria	AY004724
Luciobarbus biscariensis	El Abiod, Arris, Algeria	AY004726
Luciobarbus brachycephalus	Terek River, Russia	AY004729
Luciobarbus longiceps	Tiberias Lake, Israel	AF145942
Luciobarbus esocinus	Tigris River, Diyarbakir, Turkey	AF145934
Luciobarbus rajanorum mystaceous	Keban Dam Lake, Euphrates R, Turkey	AF145938
Luciobarbus xanthopterus	Tigris River, Diyarbakir, Turkey	AF145939
Luciobarbus graecus	Kiffisos River, Greece	AF090786
Luciobarbus mursa	Arax River, Armenia	AF145943

Table 2S.Genetic distances for complete *cytb* gene. Uncorrected genetic distances between populations are presented below diagonal. In the diagonal uncorrected distances within population are shown.

	VZ	GQ	GH	GU	PO	VR	GM	PA	GN	GD	QQ	GO	SX	MI	MV	GT	GS	ML	CM	RE	OL	SG
VZ	**0.01**																					
GQ	0.59	**0.23**																				
GH	0.11	0.68	**0.17**																			
GU	0.83	0.68	0.93	**0.6**																		
PO	0.05	0.63	0.14	0.87	**0.08**																	
VR	0.15	0.72	0.16	0.97	0.18	**0.06**																
GM	0.19	0.76	0.19	1.02	0.23	0.05	**0.02**															
PA	0.28	0.85	0.27	1.1	0.32	0.13	0.11	**0.02**														
GN	0.09	0.67	0.19	0.92	0.13	0.23	0.28	0.36	**0**													
GD	0.1	0.5	0.19	0.75	0.14	0.23	0.28	0.37	0.18	**0.01**												
QQ	0.09	0.5	0.19	0.74	0.13	0.23	0.28	0.36	0.18	0.01	**0**											
GO	0.16	0.56	0.25	0.81	0.2	0.29	0.34	0.43	0.24	0.07	0.07	**0.08**										
SX	0.09	0.5	0.19	0.74	0.13	0.23	0.28	0.36	0.18	0.01	0	0.07	**0**									
MI	0.11	0.52	0.21	0.76	0.15	0.25	0.29	0.38	0.19	0.02	0.02	0.08	0.02	**0.03**								
MV	1.02	0.79	1.12	0.75	1.06	1.16	1.21	1.29	1.11	0.94	0.93	1	0.93	0.95	**0.84**							
GT	0.55	0.4	0.64	0.4	0.58	0.68	0.73	0.81	0.63	0.46	0.45	0.52	0.45	0.47	0.6	**0.05**						
GS	0.27	0.84	0.26	1.09	0.31	0.12	0.07	0.19	0.35	0.36	0.35	0.42	0.35	0.37	1.3	0.8	**0**					
ML	1.5	1.38	1.59	1.08	1.54	1.63	1.68	1.76	1.58	1.41	1.4	1.47	1.4	1.42	1.2	1.3	1.7	**0**				
CM	1.51	1.39	1.61	1.1	1.55	1.65	1.7	1.78	1.59	1.42	1.42	1.48	1.42	1.44	1.2	1.3	1.8	0	**0.02**			
RE	0.53	0.89	0.59	0.97	0.57	0.56	0.58	0.66	0.61	0.57	0.56	0.63	0.56	0.58	1.1	0.9	0.6	1.1	1.1	**0.77**		
OL	0.48	0.37	0.58	0.61	0.52	0.62	0.67	0.75	0.56	0.39	0.39	0.45	0.39	0.41	0.8	0.3	0.7	1.3	1.3	0.8	**0.06**	
SG	0.94	0.72	1.03	0.99	0.98	1.08	1.12	1.21	1.02	0.85	0.85	0.91	0.85	0.86	1.1	0.8	1.2	1.5	1.5	1.2	0.6	**0.45**

Table 3S. Genetic distances for S7II gene. Uncorrected genetic distances between populations are presented below diagonal. In the diagonal uncorrected distances within population are shown

	VZ	GQ	GH	GU	VR	GM	PA	GN	GD	QQ	GO	MV	SX	MI	GS	ML	CM	RE	GT	OL	SG
VZ	**0**																				
GQ	0.3	**0.4**																			
GH	0	0.3	**0**																		
GU	0.3	0.4	0.3	**0.15**																	
VR	0.1	0.3	0.1	0.2	**0.14**																
GM	0.1	0.3	0.1	0.3	0.1	**0.15**															
PA	0	0.3	0	0.3	0.1	0.1	**0**														
GN	0	0.3	0	0.3	0.1	0.1	0	**0**													
GD	0.2	0.4	0.2	0.4	0.3	0.3	0.2	0.2	**0.36**												
QQ	0	0.4	0	0.3	0.1	0.1	0	0	0.2	**0**											
GO	0	0.6	0	0.3	0.1	0.1	0	0	0.2	0	**0.085**										
MV	0.2	0.4	0.2	0.4	0.3	0.3	0.2	0.2	0.4	0.2	0.3	**0.36**									
SX	0.3	0.6	0.3	0.6	0.4	0.4	0.3	0.3	0.5	0.3	0.4	0.5	**0.30**								
MI	0.3	0.4	0.3	0.2	0.2	0.3	0.3	0.3	0.4	0.3	0.3	0.4	0.6	**0.21**							
GS	0	0.4	0	0.3	0.1	0.1	0	0	0.2	0	0.0	0.2	0.3	0.3	**0**						
ML	0.4	0.4	0	0.1	0.2	0.3	0.4	0.4	0.5	0.4	0.4	0.5	0.7	0.2	0.4	**0.11**					
CM	0.1	0.3	0.1	0.2	0.1	0.1	0.1	0.1	0.3	0.1	0.1	0.3	0.4	0.2	0.1	0.2	**0.12**				
RE	0.2	0.4	0.2	0.3	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.3	0.5	0.3	0.2	0.3	0.2	**0.2**			
GT	0.1	0.4	0.1	0.3	0.2	0.2	0.1	0.1	0.3	0.1	0.1	0.3	0.4	0.3	0.1	0.4	0.2	0.2	**0.18**		
OL	0	0.3	0	0.3	0.1	0.1	0	0	0.2	0	0	0.2	0.3	0.3	0	0.4	0.1	0.2	0.1	**0**	
SG	0.2	0.3	0.2	0.1	0.1	0.2	0.2	0.2	0.3	0.2	0.2	0.3	0.5	0.2	0.2	0.2	0.1	0.2	0.2	0.2	**0.04**

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5.CHAPTER IV

This chapter is based upon the manuscript:

Casal-López & Doadrio, I., The Messinian imprint on the evolution of freshwater fishes of the genus *Luciobarbus* Heckel, 1843 (Teleostei, Cyprinidae) in the western Mediterranean.

THE MESSINIAN IMPRINT ON THE EVOLUTION OF FRESHWATER FISHES OF THE GENUS LUCIOBARBUS Heckel, 1843 (TELEOSTEI, CYPRINIDAE) IN THE WESTERN MEDITERRANEAN

ABSTRACT

The current Mediterranean Basin is the result of a complex geological history during the Cenozoic when the western Tethys was closed and reopened after the Messinian Salinity Crisis. The abrupt refilling of the Mediterranean Basin at the end of Messinian had catastrophic consequences for the present richness and distribution of freshwater organisms. Currently the genus *Luciobarbus* is the only representative of primary freshwater fishes widely distributed in the Mediterranean Basin, showing its highest diversity in the Western Mediterranean. Our results suggest that the two western lineages of *Luciobarbus* originated during the Messinian, one including most of the North African species from Morocco and western Algeria and another composed by Iberian, one Moroccan species from Moulouya Basin and eastern Algerian basins. The history of the genus at the Western Mediterranean has been strongly influenced by tectonic dynamics of the Betic-Rifian Arch and the reopening of the Mediterranean Basin. Our timing shows two main processes during Messinian that gave origin to the extant species of the western Mediterranean: one associated with the Atlantic-Mediterranean gateways and another associated with the end of the Messinian Salinity Crisis.

Introduction

The Mediterranean Basin has been traditionally considered a major biodiversity hotspot characterized by high levels of endemism, due partly to recent diversification processes and ~~partly~~ to the conservation of taxa with ancient distributions that have not undergone evolutionary changes (Myers *et al.*, 2000; Thompson 2005; Magri *et al.*, 2007). One of the explanations giving this region its characteristic species-richness lays on its geo-climatic history and on its physiographic heterogeneity (Blondel and Aronson, 1999). The Mediterranean Basin is located at the intersection of the African and Eurasian plates, a highly active tectonic area that has underwent several events through time. These events have repeatedly connected and fragmented both continents acting as main drivers of local diversification (Sanmartín, 2003, Rosenbaum *et al.*, 2002,). As consequence of plate tectonics the MB was dried out during the Messinian Salinity Crisis (~5,9 Ma) which ended with the Zanclean Flood (ZF) (5,3 Ma), when the Strait of Gibraltar reopened and the Mediterranean Basin was again refilled (Krijgsman *et al.*, 1999, 2000, García-Castellanos and Villaseñor, 2011, García-Castellanos *et al.*, 2015). Nonetheless, the complete dissecation of the MB and its fast and catastrophic refilled that has been proposed by some authors (McKenzie, 1980, 1999, Krijgsman *et al.*, 1999, Govers, 2009, Garcia-Castellanos *et al.*, 2009, Crespo-Blanc *et al.*, 2016) is still under debate. An alternative hypothesis suggests a more mild history where the Mediterranean was not entirely dried out (Stoica *et al.* 2016). These two proposed scenarios would have led to different biogeographic histories of the freshwater ichtyofauna since its dispersion is only possible through freshwater bodies.

This paleo-geographic background is important to understand the processes that allowed the establishment of connections through time between southern Iberia and northern Africa. At the western region of the MB the interaction between the Eurasian and African plates and the independent motion of the Alboran Domain during the Alpine orogeny in the late Cretaceous created the Betic-Rif arc that rims the Mediterranean Sea from southern Spain, across the Gibraltar arc, and into Morocco (Chalouan *et al.*, 2008, Vergés and Fernandez, 2012,Neres *et al.* 2016, González-Castillo, 2015). Most of the present day relief has been formed since the Miocene modulating the physiography of the landmasses constrained within this area (Dewey *et al.*, 1989, Garcia-Dueñas *et al.* 1992, Maldonado *et al.*, 1999, Gutscher et al, 2002, Braga et al, 2003, Potter and Szatmari, 2009, Pérez-Asensio *et al.* 2012, 2013, Flecker, 2015). During the Tortonian (~ 11.6 to 7.2 Ma) and before the onset of the MSC, the progressive closure of the Mediterranean–Atlantic marine connections created land-bridges that permitted terrestrial faunal exchange between Africa and southern Iberia until ~ 6.2 Ma ~~(Agustí *et al.* 2006, Garcés et al, 1998, Casanovas-Vilar *et al.* 2011, Gibert *et al.*, 2013, García-Alix *et al.*, 2016)~~ but is unknown whether they also allowed the exchange of freshwater fish. The formation of the Atlantic-Mediterranean gateways during this period could have given origin to vicariant processes in organisms such as primary freshwater fishes that present low dispersal ability.

These patterns of northern-southern intercontinental isolation and passage are likely to be accounted for the origin and spatial structuring of its endemic taxa by promoting or constraining dispersal, vicariance and extinction events (Pratt *et al.*, 2016, Platt *et al.*, 2013, Negro *et al.*, 2008, Jones *et al.*, 2006, Krijgsman *et al.*, 1999, 2000). Therefore, North Africa and the Strait of Gibraltar have been the focus of many biogeographical studies (Sanmartín, 2003, Rosenbaum *et al.*, 2002, Novo *et al.*, 2015, Huesemann *et al.*, 2014, Condamine *et al.*, 2013, Bidegaray-Batista, 2011).

Geological changes are thought to be important factors driving speciation (Mayr 1942; Hewitt 2000, Nielson *et al.* 2001; Brant and Ortí 2003; Carstens *et al.* 2004; Funk *et al.* 2005; Steele *et al.* 2005; Kozak *et al.*2006). Thus, in a changing Miocene scenario these alterations are expected to have a direct impact on the freshwater network and, consequently, on the phylogeographical structure of freshwater dependent organisms such as amphibians or fishes that present low vagility (Berendzen *et al.*, 2003, Lemmon *et al.*, 2007, Kuchta *et al.* 2016).

To provide novel insights into these issues we studied the evolutionary history of the populations of the genus *Luciobarbus* (Actynopterigii, Cyprinidae), a group highly distributed on both sides of the Strait of Gibraltar. The study area comprises the basins of southern Iberia and the basins within the Betic-Rifean Massif including ~~the~~ the Kabilian Mountains where the species are distributed. A previous study ~~(Machordom and Doadrio, 2001)~~ showed that the populations of *L. setivemenesis* from North African Kabilies Mountain area and the *Luciobarbus* populations from southern Iberian Peninsula were connected. Some recent taxonomic revisions and the description of new North African species of the genus *Luciobarbus* Heckel, 1843 based on morphological and genetic analysis (Casal-Lopez *et al.*, 2015, Doadrio *et al.*, 2016), should be taken into account in order to illustrate the phylogeographical relationships between the populations inhabiting both sides of the Strait of Gibraltar and to further explore the biogeographical history of the these cyprinid fishes.

We aim to: (1) test whether the Messinian connections between southern Iberia and Northern Africa played a key role on the diversification of the lineages of *Luciobarbus*, (2) Reconstruct the biogeographical history of the group and the events that led to their current distribution

MATERIAL AND METHODS

Taxon sampling, DNA extraction and molecular data

Fish were captured by electrofishing and fin clipped with the permission for sampling was obtained from the appropriate authorities. Fin clips were preserved in 96% ethanol and later kept at 4 ^{0}C. A small number of specimens from each sampling site were formalin preserved as voucher specimens. Both DNA samples and specimens were deposited in the DNA and Fish Collection at the national Museum of Natural Sciences (MNCN-CISC).

For phylogenetic analyses we used all the Iberian and North African species of *Luciobarbus* within the western Mediterranean Basin: *L. bocagei, L. comizo, L. graellsii, L. guiraonis, L. microcephalus* and *L. sclateri*, *L. guercifensis, L. maghrebensis, L. rabatensis, L. rifensis, L. ~~moulouyensis~~, L. setivamensis* and *Luciobarbus sp*. from Chiffa. *Barbus petenyi* was used as outgroup. We also included *L. brachycephalus* as representative of the eastern Mediterranean lineage (Table 1S).

For phylogeographic networks different populations of different drainages inhabiting the Betic-Rifian Arc region were compared (Fig.1) (*L. sclateri*, *L. guercifensis, L. maghrebensis, L. rabatensis, L. rifensis, L. yahyaouii, L. setivamensis* and *Luciobarbus sp*. from Chiffa).

For the estimation of divergence times we opened the phylogeny to maximize the number of calibration points available we included DNA sequences from species of the genus *Luciobarbus,* both from western and eastern lineages including those of which fossil record is known: the oldest known *Luciobarbus* fossil dated to 10.5 Ma (Boehme, 2002) and *Luciobarbus sp*. from the Iberian Peninsula dated ~6 Ma considering the MEIN13 (Doadrio and Casado, 1989, Sesé, 1989, García-Alix *et al.*, 2008). Three species from *Barbus* genus and one species from *Aulopyge* genus were chosen as outgroups (Table 1S).

Total genomic DNA of 323 individuals was extracted using the commercial kit Biosprint15 for tissue and blood (Qiagen). Two molecular markers were amplified: the complete mtDNA cytochrome *b* gene (*mt-cytb;* 1140 bp) and a fragment of the intron 1 of nuclear gene actin (*n-actinII*; 489 bp). Primers and protocols for the amplification are presented in Table 2. After checking PCR products on 1% agarose gels, they were purified by ExoSAP-IT™ (USB, Cleveland, USA) and directly sequenced by MACROGEN Inc (Amsterdam, The Netherlands; http://www.macrogen.org) using a 3730XL DNA Sequencer.

Table1. Primers and Protocols PCR

Gene	**Primer name**	**Sequence 5'-3'**	**Tann (C^0)**	**Source**
cytb	GludGL	TGACTTGAAR AACCA YCGTGG	48	(Palumbi,1996)
	H16460	CGAYC TTCGG ATTAA CAAGA CCG		(Doadrio & Perdices, 2001)
actin-II				(Markova *et al*, 2010)

Sequences were aligned using the default pairwise and multiple parameters in Clustal W implemented in MEGA software v 5.1. Chromatograms and alignments were revised and visually adjusted. We found in *actinII* PCR-amplified mixtures of two products that differ in length, corresponding to heterozygous individuals for several insertions or deletions (*indels*). This constrained the use of the software commonly used

for phase data and thus, alleles were manually phased using the method described by Sousa-Santos *et al.*, 2005; Flot *et al.*, 2006. Recombination of the nuclear *actinII* gene (p = 0.539) was tested using the PHI test in Splitstree v. 4.13 (Huson and Bryant, 2006).

Phylogenetic analyses

For *mt-cytb* and *n-actinII* genes we used jModeltest 0.1.1 (Posada, 2008) to estimate the evolutionary model that best fitted the data for each gene separately, using AIC (Akaike information criterion).

For *cytb* the best model was GTR+G (rate matrix: R(a)[AC] = 0.6119; R(b)[AG] = 20.5122; R(c)[AT] = 0.3024, R(d)[CG] = 0.8655; R(e)[CT] = 6.4844; R(f)[GT] = 1.00; gamma shape = 0.2590). For *actinII* the best model scheme was TIM3+I (rate matrix: R(a)[AC] = 3.003; R(b)[AG] = 2.6205; R(c)[AT] = 1.000, R(d)[CG] = 3.003; R(e)[CT] = 5.3684; R(f)[GT] = 1.00; gamma shape = 0.7300).

Bayesian inference (BI) was performed using MrBayes v.3.2 (Ronquist *et al.*, 2012). Two simultaneous analyses were run for 10^7generations, each with four MCMC chains sampling every 100 generations. Convergence was checked with Tracer v.1.6. (Rambaud *et al.*, 2014). After discarding the first 10% of generations as burn-in, the 50% majority rule consensus tree and posterior probabilities were obtained. Phylogenetic analyses of *mt-cytb* gene independently and *mt-cytb* and *actinII* concatenated were conducted using Maximum Likelihood (ML) in RaxML software implemented in the Trex-online server using the substitution model GTRGAMMA and the rapid bootstrap algorithm (Stamakis, 2006) for both *mt-cyb* and concatenated analyses. Node confidence in ML analyses was estimated by rapid bootstrapping using a random seed (1000 replicates).

Gene networks

To assess the phylogeographic history of *Luciobarbus* in the Strait of Gibraltar, haplotype networks for *mt-cytb* and *n-actinII* were constructed by using a median joining algorithm (Bandelt *et al.*, 1999) and edited in the software Network 4.5.1.6 (http://www.fluxus-engineering.com).

Mitochondrial genes showed large divergence and it was analysed using Splitstree4 4.10 (Hudson & Bryant, 2006). Default settings were used to construct a neighborNet with uncorrected p-distances.

Divergence times estimates

For *mt-cytb* we inferred a time-calibrated phylogeny in BEAST v1.8.2 (Drummond AJ, Suchard MA, Xie D & Rambaut A, 2012) using a lognormal relaxed clock, following a Birth-Death model of speciation and using fossil calibration. Markov Montecarlo chains were run for 50^6 generations with a sampling frequency of 10 000, and Tracer v 1.6 (Rambaud *et al.*, 2014) was used to assess convergence. The effective sample sizes for all parameters of interest were greater than 200. Finally, the trees were summarized with the software TreeAnnotator v.1.7.2 (Drummond *et al.*, 2012) to obtain a maximum clade credibility tree with estimated divergence times.

Biogeographical Reconstructions

We used the same *Luciobarbus* species that were used in the phylogenetic analyses without the outgroup *Barbus petenyi* and the eastern representative *L. brachycephalus* reconstructed the ancestral states of the distribution ranges of the populations of the genus *Luciobarbus* on both sides of the Strait of Gibraltar, using a dispersal-extinction-cladogenesis (DEC) model of LAGRANGE (Ree&Smith, 2008) implemented in RASP 2.1 (Yu *et al.*, 2014). The dichotomous and ultrametric trees for separate *mt-cytb* and *n-actinII* datasets were obtained in BEAST 1.7 (Drummond *et al.*, 2012) and were used as the tree topology on which mapping ancestral areas. For both genes, a Birth-Death model was set as tree prior. A minimum

calibration point was set as tree root, by taking into account the most recent common ancestor (MRCA) of *Luciobarbus* (10.5 Ma, Boehme, 2002). A normal prior was assigned to the ucld.mean parameter of the lognormal relaxed clock for both *mt-cytb* and *n-actinII*. For *mt-cytb* the initial and mean value was 0.76% per site per million year as the evolutionary rate proposed for European cyprinids (Perdoya and Doadrio 1999) and 0.1 value for standard deviation. For *n-actinII*, the default parameters of the normal distribution were usedt. MCMC analyses were run for 20 million generation, with parameters logged every 1000 generations. The remaining parameters were used as default. Output from BEAST 1.7 was examined in Tracer 1.6 and the results were summarized using TreeAnnotator1.7 (Drummond *et al.* 2012). Tips of the ultrametric tree were coded as the current distribution areas of each species of *Luciobarbus*.

Diversification

In order to test changes in the diversification rates through the evolution of the groups we used a log-line-age through time approach LTT (Barracough & Nee, 2001). We used the R library 'ape' (Paradis *et al.,* 2004) using the *mt-cytb* and *n-actinII* ultrametric trees obtained in BEAST. LTTs represent graphically the time elapsed between successive branching events (Barraclough and Nee, 2001). The ultrametric tree contains information on the number of lineages and the molecular distance of every lineage to the root (the relative time of each node from the root node). We used the y-statistic (Pybus & Harvey, 2000) to test for temporal shifts in diversification rate.

RESULTS

Phylogenetic analyses

The resulting matrix of the dataset consisted of 323 specimens and 1140 characters corresponding to *mt-cytb*: 1140 characters and dataset for *n-actinII* consisted of 489 characters. Mitochondrial phylogenetic tree supported the split in two main lineages, one comprising North African species with Caucasian (*L. brachycepahlus*) and a second one, constituted by Iberian species (Fig. 2). Two exception were *L. guercifensis* and *L. setivimensis* that suprignsly were clustered with the Iberian group. Within North African lineage, the western species *L. rabatensis*, *L. rifensis* and *L. maghrebensis* grouped together, where *L. rabatensis* and *L. maghrebensis* were sister group to *L. rifensis*. The relationships of the eastern species *L. moulouyensis* and *L. setivimensis* from the Mediterranean slope were unresolved. The Asiatic *L. brachycephalus* was basal to North African species (Fig. 2).

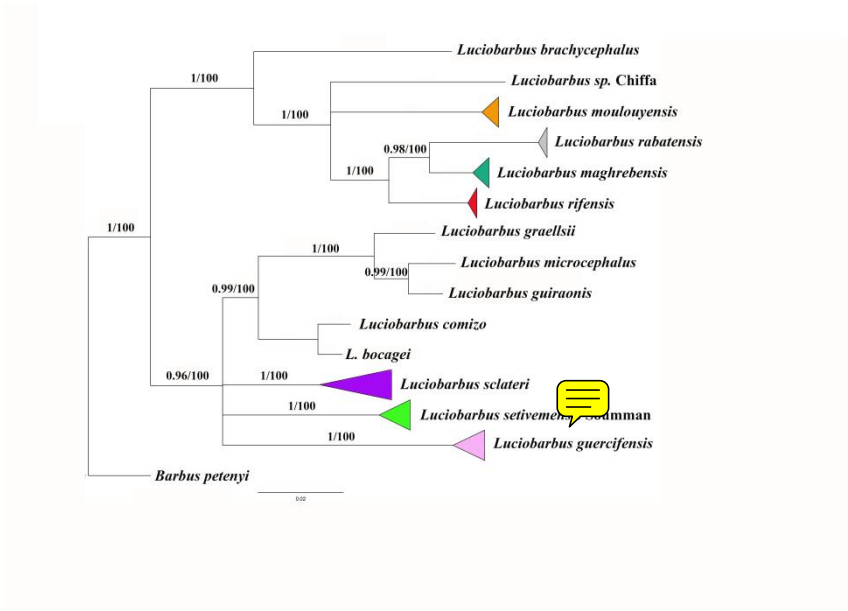

Fig.2. Phylogenetic tree of *cytb* gene rendered by Bayesian Inference (BI). Numbers on branches indicate posterior probability values for BI and under branches indicate bootstrap for ML.

Nuclear gene, due to a lower number of characters showed poorly resolved relationships although there is some structuring in some groups. We recovered the North African lineages without *L. brachycephalus*, which is clustered with Iberian species. Moreover, discrepancies were found in *L. setivimensis* which in nuclear phylogeny clustered with geographically close North African species and not with the Iberian as it happened in the mitochondrial relationship. Iberian species are show low values of support but the presence of north Afrcian *L. guercifensis* should be highlighted since this relationship is also found in the mitochondrial phylogenetic tree (Fig.3).

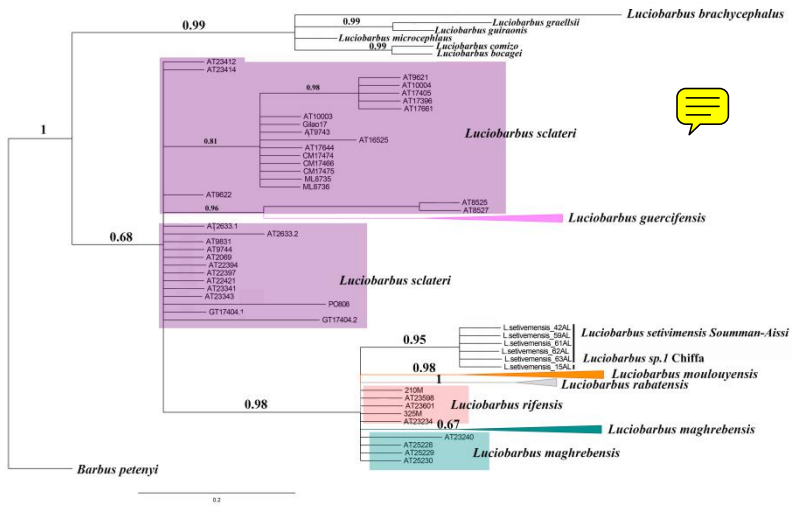

Fig.3. Phylogenetic tree of *actinII* gene rendered by Bayesian Inference (BI). Numbers on branches indicate posterior probability values for BI.

Gene networks

Networks for *mt-cytb* showed a clearer differentiation than the mitochondrial phylogenetic tree (Fig.4). As for the *actin-II* network, it was ~~much~~ more informative than the phylogenetic tree. Interestingly we found two groups, one comprised by Iberian species *L. sclateri* and North African *L. guercifensis* and another one constituted by the rest of North African species. Within the North African group, *L. rabatensis* showed a unique nuclear haplotype. The Algerian species, *L. setivemesis* and *L.sp* from Chiffa showed a unique and shared haplotype for both species. *L. maghrebensis* show more number of nuclear haplotypes from which two of them were shared with geographically close *L. rifensis* and *L. ~~moulouyensis~~* from Morocco (Fig.5).

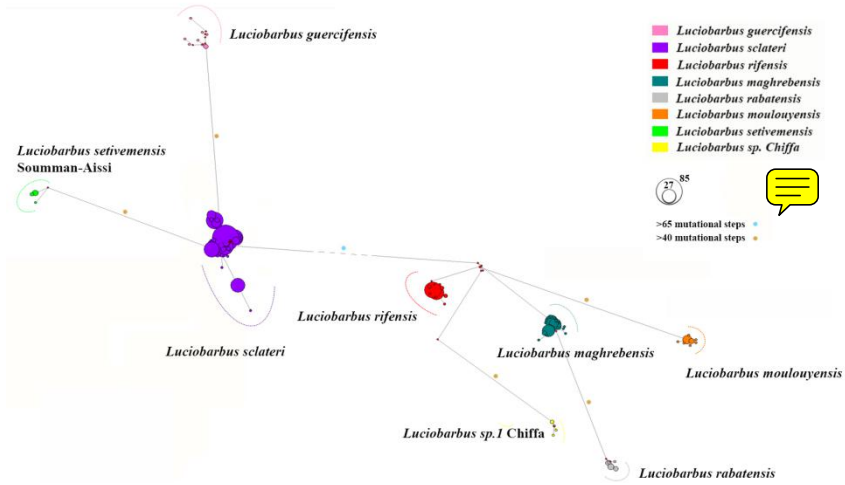

Fig.4. *Cytb* haplotype network. Mutations are indicated with numbers on branches linking haplotypes. Legend of colours shown corresponds to the species of *Luciobarbus* represented. Circle size represent the number of individuals and blue and orange dots correspond to the number of mutational steps.

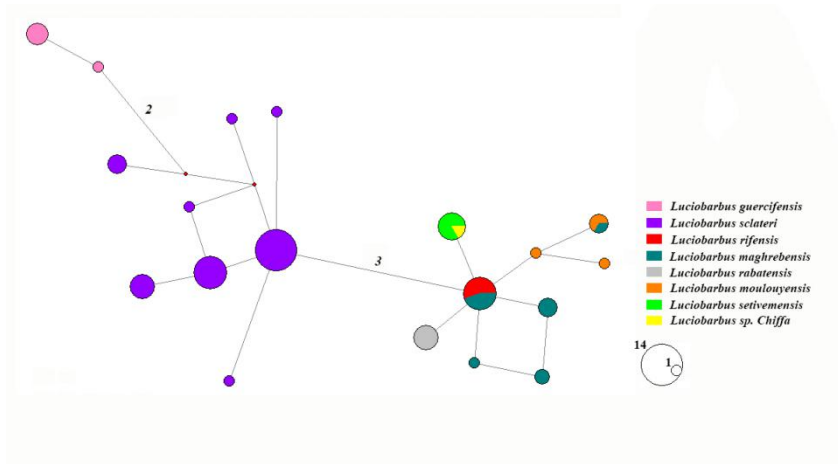

Fig.5. *actinII* haplotype network. Mutations are indicated with numbers on branches linking haplotypes. Legend of colours shown corresponds to the species of *Luciobarbus* represented. Circle size represent the number of individuals and blue and orange dots correspond to the number of mutational steps.

Estimation of ages of divergence and diversification

In this opened phylogeny counting with a higher number of species resulted in phylogenetic relationships ~~much more~~ ~~well supported~~. The split of Mediterranean *Luciobarbus* into two major clades, comprised by lineage A formed by Iberian *Luciobarbus* species and *L. guercifensis* and *L. setivemensis*; and a lineage B, comprised b~~u~~y North African and Asian species was estimated to have occurred in the Miocene around 11 Ma (95% HPD:9.6-12.7). Within lineage A the North African *L. guercifensis* split around 6.7 Ma (95% HPD: 7.6-5.9) from the rest of the groups. The rest of the groups are divided into two clades that were separated around 6.2 Ma (95% HPD: 7.1-5.5 Ma). The Algerian species within the Iberian species clustered with a second group that was separated around 5.3 Ma (95% HPD 5.2-5.4 Ma). Lineage B underwent a first split around 9.6 Ma (95% HPD: 8.3-11 Ma) giving origin to North African and Asian clades. Around 7.1~~2~~ Ma (95% HPD: 8.7-5.6 Ma) *L. ~~moulouyensis~~* split from the rest of North African species and followed by a second split between North African and Algerian species around 5.9 Ma (95% HPD: 7.4-4.5 Ma). The North African species around the Strait of Gibraltar were divided during Pliocene. As for the Asian lineage split around 7.2 Ma (95% HPD= 8.4-6-2 Ma), respectively gave origin to the extant species of each lineages (Fig.6).

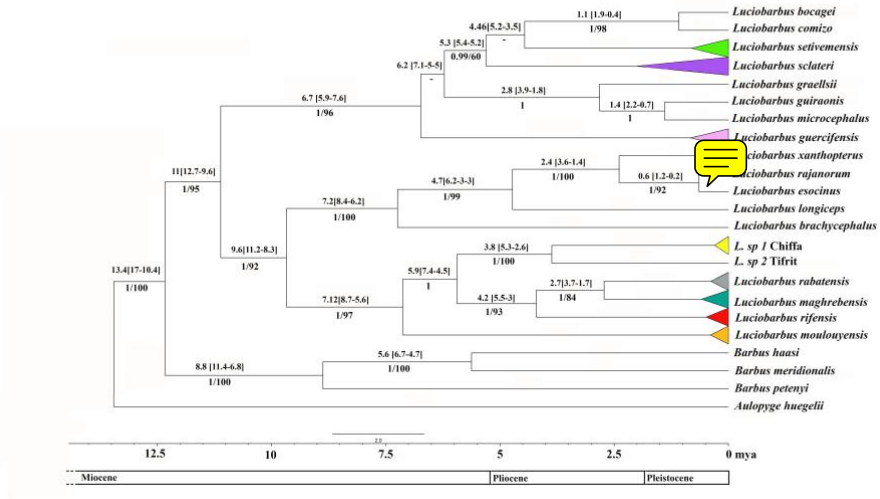

Fig 6.- Calibration tree and divergence times based on *cytb* gene and estimated through fossil record for the species of the genus *Luciobarbus*. Numbers, above and under branches, represent divergence ages estimation and their HPD 95% confidence intervals, respectively.

The LTT plot (Fig. 7) reflects the temporal pattern of diversification of *Luciobarbus* species showing the number of lineages over time. For *mt-cytb* the diversification rate stays steady from 10 Ma to almost 6 Ma, with no increase in the number of lineages in this period. From this point on, the number of nuclear lineages begins to increase. As for *n-actinII*, a constant rate is maintained over time. Interestingly, both markers show a considerable increased of the diversification rate from 2.5 Ma, during the Plio-Pleistocene.

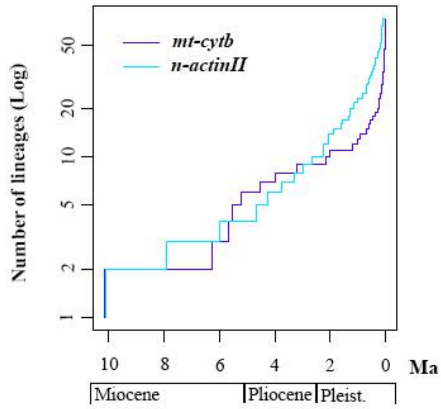

Figure 7. Lineage through time plot (LTT) obtained from the ultrametric tree in Fig. 6.

Ancestral area reconstruction

For *mt-cytb* the reconstruction using LAGRANGE (DEC) method showed a biogeographical history followed by *Luciobarbus* species in which dispersal and vicariant events were frequent (Fig 8). In total, DEC analysis detected 14 dispersals, 12 vicariance events and 1extinction for *mt-cytb*. As diagrams show most of dispersion events are detected in a recent period of time (Pleistocene), whereas most of the vicariant events occurred during upper Miocene and Pliocene, coinciding with the Messinian Crisis and posterior refilling of the ~~MB~~ during the ZF (Fig ~~XX~~). Two main lineages were found: lineage A, distributed mainly across the Iberian Peninsula and two small areas of North Africa (~~-~~reduced to Moulouya Basin, Soumman and Aissi basins), and another one, lineage B, widely distributed in North Africa.

Nonetheless, within the lineage A, DEC was not able to resolve the state of the ancestral node. The separation of North African basins from the Iberia peninsula occurred in two different periods of times driven by vicariant events. The first one, in the upper Miocene, corresponded to Moulouya Basin in Morocco, where *L. guercifensis* is currently distributed. Another vicariant event during the ~~Plio-Pleistocene~~ resulted in the separation of

Soumman and Aissi basins in Algeria where *L. setivemensis* show its present-day distribution, from the rest of Iberian species. Within the Iberian Peninsula, DEC estimated a series of vicariance and dispersal events during the Plio-Pleistocene resulting in the split between two biogeographic areas within Iberian species, one corresponding mainly to eastern Iberian basins (where *L. graellsi*, *L. guiraonis* and *L. microcephalus* are currently mainly distributed) and other mainly corresponding to the western Iberian basins (where *L. comizo* and *L. sclateri* have their present-day distribution areas). In North Africa, within lineage B, DEC analysis poorly resolved the common ancestral node of the North African group, although it detects a vicariant event in the upper Miocene that could be claimed as the origin of the ancestral split between the distribution of eastern and western North African populations, giving rise to the current distribution areas of the eastern species, from Moulouya Basin and Chiffa Basin, where *L. ~~moulouyensis~~* and *L. sp.* are currently distributes, respectively; and of the western species, from basins comprised between M'Ter Basin to the east and Bou Regreg Basins to the west, where *L. ghrebensis*, *L. rifensis* and *L. rabatensis* are currently distributed (Fig 8).

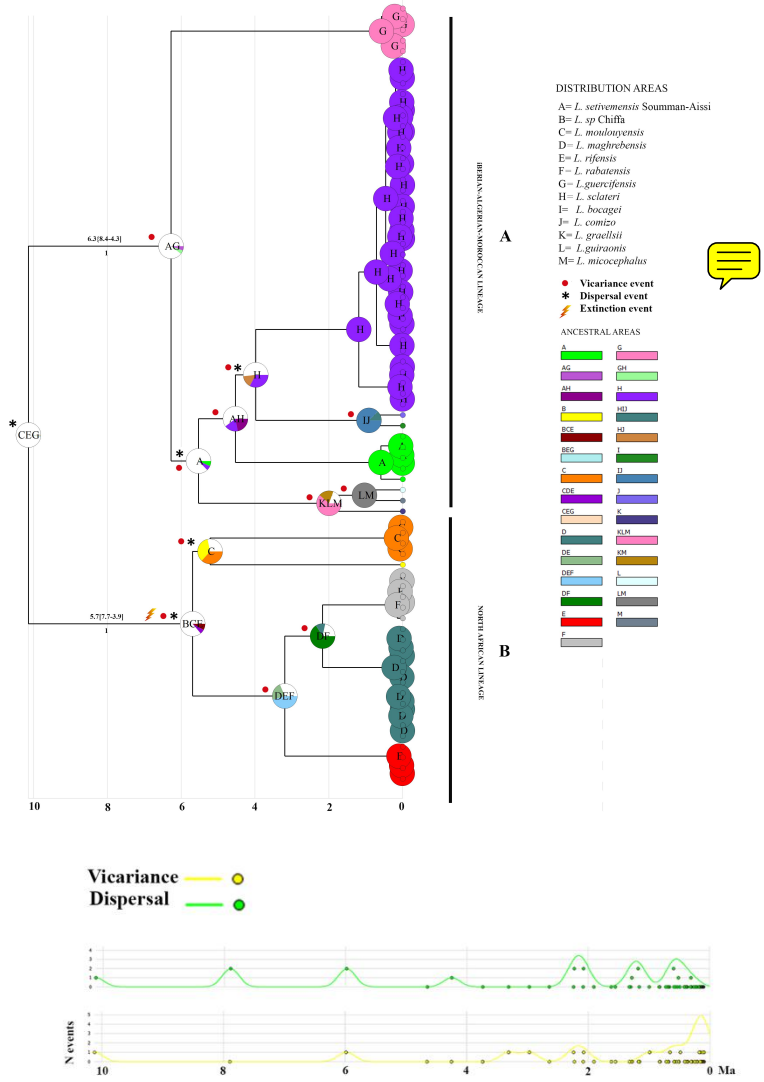

Figure 8 Ancestral area reconstruction of *Luciobarbus* populations on a *cytb* tree generated by BEAST using BayArea model implemented in RASP software. Legend of colours represents possible ancestral ranges. The Most Likely State is represented at the pie chart, with the code and colour corresponding to the area it is assigned. Diagram below shows the number of dispersal and vicariances events through time.

For *n-actin II* DEC analysis detected a different history to the mitochondrial, with a total balance of 17 dispersals and 14 vicariance events. Most of dispersion and vicariance events are detected in a recent period of time (from upper Pliocene to Pleistocene), as it can be seen in Fig 9, though some dispersion and vicariant events also occurred between upper Miocene and early Pliocene, coinciding with the main splits within Iberian and North African clades. As it was also observed in previous *n-actin II* phylogenetic and phylogeographical analyses, Soumman and Aissi basins, belonging to the present-day distribution of *L. setivamensis* clustered together with other North African areas, and not with Iberian ones. Within each of the two lineages A and B, a more complex biogeographic history due to a higher number of dispersion than that detected in the mitochondrial.

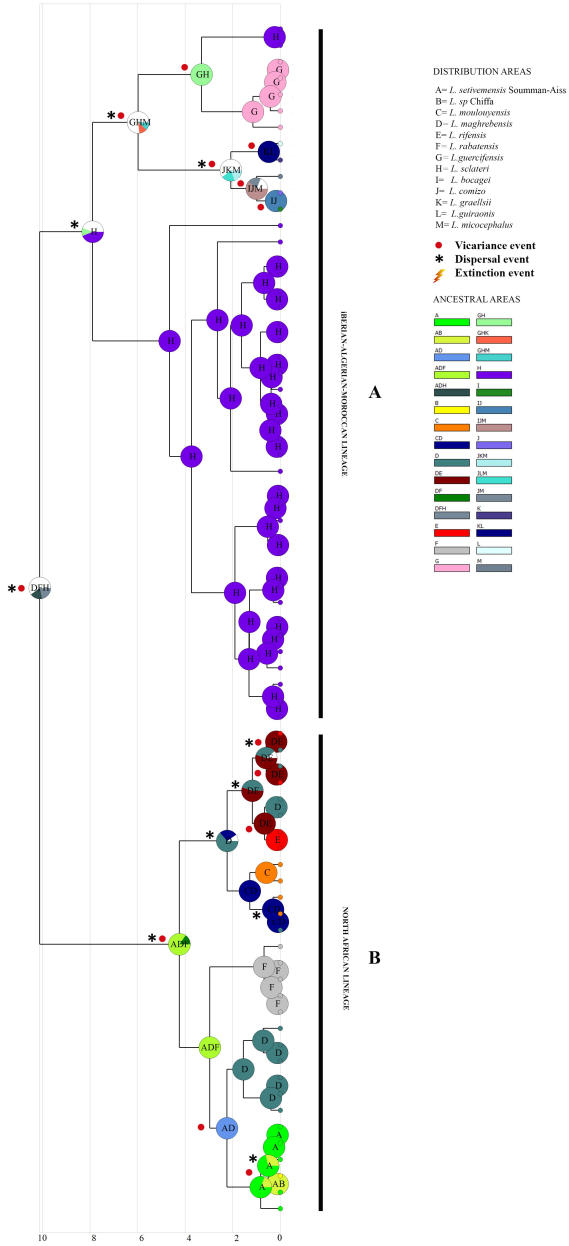

Figure 9 Ancestral area reconstruction of *Luciobarbus* populations on a *actinII* tree generated by BEAST using Dispersal-Extinction-Cladogenesis (LAGRANGE) model implemented in RASP software. Legend of colours represents possible ancestral ranges. The Most Likely State is represented at the pie chart, with the code and colour corresponding to the area it is assigned.

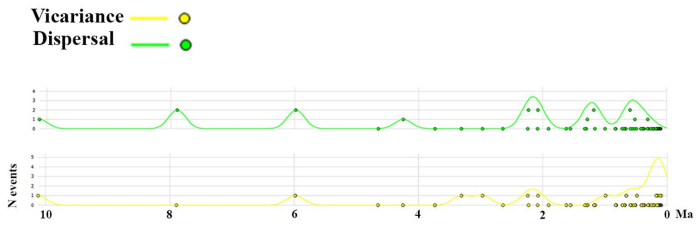

Figure 10. Diagram from the ancestral area reconstruction of *actinII* showing the number of dispersal and vicariance events through time.

DISCUSSION

The upper Miocene was an important period of changes in the Western Mediterranean with the formation of the Betic-Rifian corridors and the isolation of the Mediterranean leading to the onset of the Messinian Salinity Crisis (MSC) (around 5.96 Ma, Krijgsman *et al.*, 1999). Our dating analysis and tree topology shows an evolution of the genus *Luciobarbus* greatly influenced by the MSC. We found that the *mt-cytb* structure is better defined than that found in the *n-actinII*, likely due to a minor number of informative characters in the nuclear marker, as shown by other authors (Perea *et al.*, 2016, Kuchta *et al.*, 2016). Nonetheless, a significant discordance in both markers leads to a different story for the Algerian species *Luciobarbus setivamensis*, which currently inhabits the Soumman and Aissi basins. This discordance is likely due to the mitochondrial marker reflecting older processes than those detected through the nuclear marker (Toews and Brelsford, 2012). Our results support the existence of two different gateways between Atlantic and Mediterranean occurring in different periods during the upper Miocene that resulted in the speciation process of the extant species of *Luciobarbus.*

Miocene imprint on the evolution of Luciobarbus species

Serravallian-Tortonian

Our dating and biogeographical results show a basal split of the genus *Luciobarbus* into two main lineages A and B and a posterior dispersion during middle-upper Miocene (around 11 Ma, 95% HPD= 12.7-9.6). Previous paleogeographical studies showed that during the Serravallian regression (from 13.8 to 11.62 Ma), there was an intermittent connection between Mediterranean Sea and Indian Ocean that was finally closed at about 11-10 Ma (Rölg, 1999). This scenario probably allow a freshwater fish fauna exchange between Middle East and North Africa, which would explain the relation found between the species of genus *Luciobarbus* of the lineage B. The presence of *Luciobarbus* species in Pasalar (Turkey) deposits dated between Burdigalian and Serravallian period (17-12.5 Ma) (http://www.wahre-staerke.com/) along with the absence in North Africa during the Miocene of paleartic freshwater fish species (Greenwood, 1974) may indicate that the colonization of North Africa by *Luciobarbus* started in Middle East during upper Miocene. During this period, the formation of the Danube (Leever *et al.*, 2011) could have allowed the dispersion in central Europe of *Luciobarbus,* as supported by the presence in Vosedorf of *Luciobarbus* ~~fossils~~ dated ~~to~~ 10.5 Ma (Böheme, 2002). During this ~~epoch~~ the Mediterranean was closed and a connection was established allowing dispersal from eastern Mediterranean to the western part (Rögl, 1999, Rosenbaum, 2002. 2014). This scenario seems highly plausible with the dispersion of the genus *Luciobarbus* giving origin to the two main lineages.

The Tortonian was a period of high tectonic activity between south-eastern Iberia and North Africa ~~(Alvinerie *et al.* 1992, Martin et al, 2009, Flecker *et al.*, 2015)~~ that favoured vicariance events leading to speciation processes in different taxa (Levy *et al.*, 2009, Bidegaray-Batista and Arnedo, 2011, Faille *et al.*, 2014, García-Vazquez et al, 2016).

Messinian onset

The results from our biogeographical analyses indicated that most of the vicariant events for the genus *Luciobarbus* occurred during this period. The Messinian was a catastrophic period for the Mediterranean Basin, due to the Messinian Salinity Crisis and the posterior refilling at the end of this period. This had an effect on both lineages of *Luciobarbus*, A and B. In the case of lineage A, comprised by Iberian species and two North African species (*L. guercifensis* and *L. setivimenisis*), we found two vicariant events that indicated that the separation of Iberian Peninsula and North Africa took at least two different times during the Messinian. One of these vicariant events was the refilling of the Mediterranean Sea at 5.3 Ma (Krigjsman *et al.*, 1999, 2000). Up to date, this is the only event that has been shown to explain the difference between sister groups of freshwater fish fauna (Doadrio, 1990, Zardoya and Doadrio, 1998, Machordom and Doadrio, 2001, Perdices and Doadrio, 2001). Nonetheless, we found evidence of a previous event that split the populations from Iberian Peninsula and North Africa during the Messinian around 6.7 Ma (Fig ~~datacion~~). Previous works have suggested the existence of two main gateways that were the last connection between the Atlantic Ocean and the Mediterranean Sea around 6.9 to 6 Ma (Esteban *et al.*, 1996, Krijgsman at el. 1999b, Martin *et al.*, 2009). This diversification pattern has also been found in other taxa (Agustí *et al.*, 2006, Bidegaray-Batista and Arnedo, 2011, Faille *et al.*, 2014, García-Vazquez et al, 2016).

The present-day distribution of the species *L. guercifensis* and *L. setivimensis*, phylogenetically close to the Iberian species, on the eastern region of the Gibraltar Arc, could be related to the fast block rotation of this system (Crespo-Blanc, 2016).

During this period, within the Iberian Peninsula we found the separation of the two main extant groups of endemic *Luciobarbus*, one related to the Miocene endorheic basins Tajo and Duero, to the west (currently inhabited by *L. comizo* and *L. bocagei*), and another mainly related to the Miocene endorheic Ebro Basin, to the east (currently inhabited by *L. guiraonis* and *L. graellsii*), showing some level of overlap in the southern basins.

During the Messinian period, a first split within lineage B separated the eastern populations of Morocco (where *L. ~~moulouyensis~~* currently inhabits) from the rest around 7.12 Ma (HPD). The presence of two sympatric species in the Moulouya Basin, *L~~uciobarbus~~ guercifensis* and *L~~uciobarbus~~ ~~moulouyensis~~* one belonging to the lineage A and the other one belonging to lineage B with similar divergence times, 6.7 (95% HPD=7.6-5.9) and 7.12 (95% HPD=8.7-5.6) respectively confirm the existence of a gateway that have allowed the vicariance between North African and Iberian *Luciobarbus* species as was previously indicated. The existence of two species in the Moulouya Basin is possible due to different ecological requirements, one being rheophilic and the other being limnophilic ~~(citar);~~

Around 5.9 Ma (95%= 4.5-7.4) our biogeographical analyses show a segmentation of North Africa, dividing the Algerian basins (where *Luciobarbus sp* currently inhabits) with respect to Moroccan basins (currently inhabited by *L. maghrebensis*, *L. rabatensis* and *L. rifensis*). This scenario is in agreement with the paleogeographical reconstructions during Messinian that have shown a complex fragmentation of North Africa (Rosenbaum, 2002).

Messinian mitochondrial and nuclear biogeographical discrepancies

Mitochondrial and nuclear markers show an important discordance for *Luciobarbus* lineages when depicting the Messinian scenario: In contrast to the mitochondrial, the nuclear data show that *L. setivemensis* belongs to the North African and not to the Iberian species. We postulate that the presence of the nuclear genome of *L. sp* in the basins of Setif and Soumman is due to a posterior secondary contact. This way, mitochondrial genome might have remained in Setif and Soumman basins. This a common finding in European cyprinids species where the partial or complete presence of different mtDNA of different species occurs (Durand *et al.*, 2002). In a biogeographical context, dispersion of *Luciobarbus sp.* coming ~~from Chiffa and Tifrit basins~~ towards Soumman and Setif might have taken place.

Lago Mare Phase

The present-day distribution of Mediterranean freshwater organisms might be explained through the "Lago Mare" ~~stage~~ (Hsü *et al.* 1973,1977) which is considered to have played an essential role for the early penetration by Paratethyan primary freshwater fishes and for dispersion in peri-Mediterranean river systems (Bianco, 1990. Durand *et al.*, 2002 Tsigenopoulous *et al.*, 2003). This stage was characterized by fresh-brackish water conditions ~~(Bianco, 1990),~~ mediated by an improvement of climatic condition along with the input of freshwater from North African rivers, greatly due to the Eosahabi River ~~(Griffin, 2002, 2006)~~ and Paratethys input (Krijgsman, 2016). Under these conditions the dispersal of barbels could have been favoured since barbels show moderate levels of salinity tolerance (up to $15^{0}/_{00}$) (Kraiem & Pattee, 1988). Besides, our biogeographical analyses support this scenario detecting a peak of dispersion at the end of Messinian, which coincides with the dispersion of aquatic mammals, such as *Hippopotamus antiquus* (Aguirre, 2003).

Nonetheless, some data have lines of evidence that do not favour this dispersion model: (1) the absence of *Luciobarbus* in northern Mediterranean area from Greece to Iberian Peninsula; (2) the absence of freshwater primary fishes in the deposits of Cava Serredi (Italy) during the Lago Mare Phase (Carnevale, 2006) and; (3) the presence in the Iberian Peninsula of *Luciobarbus sp*. in Guadix-Baza deposits during the MEIN 13 before Lago Mare Phase (Doadrio and Casado, 1989. Doadrio and Carmona, 2003). Besides, in opposition to ostracods deposits (Stoica, 2016), Iberian *Luciobarbus* have no phylogenetic relationships with *Luciobarbus* from the eastern region of the Mediterranean area, as our time-calibrated tree shows. Based on the aforementioned reasons, a hypothetical short-distance dispersion of local fauna between close regions through Lago Mare would have been plausible. Nonetheless, the hypothesis of the connections and isolations through intercontineal bridges ~~proposed by Doadrio (1990) and Machordom et al, 1998~~ seems to better fit our data.

Plio-Pleistocene current distributions

Our reconstruction of the biogeographical history of *Luciobarbus* species in the western Mediterranean is in agreement with previous works that pointed out the Plio-Pleistocene as an important period in the diversification of cyprinids ~~(Zardoya and Doadrio, 1998, Robalo *et al.*, 2006, Sousa-Santos *et al.*, 2007, 2014, Almada and Sousa-Santos, 2010),~~ as well as other freshwater fauna (Ribera and Vogler, 2004, Ribera *et al.*, 2011). During this period, the current species of *Luciobarbus* were formed, highlighting the importance of this period in the species diversification processes, as is reflected by the diversification rate of the LTT plot (fig 7). During the Middle-Upper Pliocene until Pleistocene the large Iberian Miocene endorheic lagoons transformed into the current exorheic basins associated with tectonic reactivation of the region and resulted in tilting and

local deformation of Neogene formations (Calvo *et al.*, 1993 and Pais *et al.*, 2012).

However, our results reflect discordance between the information of mitochondrial and nuclear markers when depicting the biogeographical history of the species of North Africa *Luciobarbus*. Whereas in *mt-cybt* the species *L. maghrebensis*, *L. rifensis* and *L. ~~moulouyensis~~* are well delimited, some individuals belonging to *L. maghrebensis* show some identical alleles with respect to *L. rifensis* and *L. ~~moulouyensis~~* in *n-actinII* (Fig CC). This has been already proposed for the Iberian *Luciobarbus* as showing semipermeability of species boundary under sympatric conditions after a period of allopatric differentiation (Gante *et al.*, 2015). This idea cannot be dismissed since the geographical proximity of the basins where *L.maghrebensis*, *L. ~~moulouyensis~~* and *L. rifensis* are currently distributed, may have both recent connection between these areas and species gene flow. Another hypothesis fitting our results would be that we are detecting a case of nuclear ancestral polymorphism. Nonetheless, the poor resolution obtained through the nuclear marker when species are evolutionary close could be a bias in our interpretation of results. A multiple nuclear *loci* approach could be claimed as a solution to this problem, though this is a challenging question due to the polypoid condition of this group.

CONCLUDING REMARKS

Our reconstruction of the biogeographical history of *Luciobarbus* shows that the evolution of the group has been greatly shaped by geological changes since Miocene. These changes have caused the dispersion and vicariance of the main lineages and show the importance of the configuration of Mediterranean Basin for the fish fauna and the key role played by the opening and closure of the different gateways that allowed connections between the Mediterranean and the Atlantic and Red Seas in a

dynamic process through time. These gateways promoted vicariance events that allowed dispersion and speciation of isolated population.

The western area of Mediterranean had a complex geological history. As consequence, a series of complex biogeographical patterns have been reflected in the history of the genus *Luciobarbus*. During the Messinian we detect two important vicariant processes that would explained the presence in North Africa of two species of Iberian origin: a first one, at the beginning of the Messinian and a second one at the end of the same period, with the refilling of the Mediterranean and the establishment of the present-day configuration of the Gibraltar Arc. Nonetheless, the current distribution of *L. guercifensis* and *L. setivamensis*, at eastern Morocco and eastern Algeria, respectively, could be due to the rotation undergoing since 9 Ma by the blocks comprising the Gibraltar Arc.

Most of the speciation observed in our group has occurred in the Pliocene when the Iberian Peninsula begins to change from endorheic to an exoreic scenario, with the beginning of the present-day freshwater systems. In the North Africa, it is likely to have undergone a similar process in its freshwater network, causing a similar pattern of speciation within the North African species. Posterior to Pliocene, secondary contact between basins in North Africa are the more plausible to explain the signal found in nuclear and mitochondrial genes between species.

Supplementary material

Table 1S.Outgroups and species within the genus *Luciobarbus* included in phylogenetic analysis and molecular clock.

Current species assigantion	Freshwater system	Country
Luciobarbus maghrebensis	Beth River	Morocco
	Ouehrra River	
	Kanar River	
	Mter River	
	Drader River	
	Bouchia River	
	Ifrane River	
Luciobarbus rabatensis	Grou River	Morocco
Luciobarbus rifensis	Hachef River	Morocco
	Hajera River	
	Laou River	
	Loukos River	
	Zandoula River	
Luciobarbus yahyaouii	Zobzite River	Morocco
	Moulouya River	
	Barred River	
Luciobarbus guercifensis	Melloulou River	Morocco
	Moulouoya River	
Luciobarbus setivemensis	Aissi River	Algeria
	Soumman River	
Luciobarbus sp.1	Chiffa River	Algeria
Luciobarbus sp.2	Tifrit River	Algeria
Luciobarbus graellsii	Arga River	Spain
Luciobarbus microcephalus	Zújar River	Spain
Luciobarbus guiraonis	Mijares River	Spain
Luciobarbus comizo	Guadalmez River	Spain
		Portugal
Luciobarbus bocagei	Ibor River	Spain
		Portugal
Luciobarbus sclateri	Guadalquivir River	Spain
	Guadiana River	Portugal
	Odiel River	
	Guadiaro River	
	Barbate River	
	Guadalete River	
	Miel River	
	Manilva River	
	Guadalmansa River	
	Verde River	
	Guadalhorce River	
	Vélez River	
	Posadas River	
	Padrón River	
	Segura River	
	Gilão River	
Luciobarbus brachycephalus	Terek River	Russia
Luciobarbus esocinus	Dicle 5 km west of Hasankeyf	Turkey
Luciobarbus longiceps	Yarmuk River	Syria
Luciobarbus xanthopterus	Tigris River	Turkey
Luciobarbus mystaceus	Keban Dam Lake (Euphrates R)	Turkey
Barbus haasi	Ebro RIver	Spain
Barbus meridionalis	Besos River	Spain
Barbus petenyii	Ialomita River	Romania
Aulopyge huegelii	Busko Lake	Bosnia and Herzegovina

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6. CHAPTER V

This chapter is based upon the manuscript:

Casal-López & Doadrio, I., A phylogeny revised of the genus *Luciobarbus* (Heckel, 1843) (Actinopterygii, Cyprinidae)

A PHYLOGENY REVISED OF THE GENUS *LUCIOBARBUS* (HECKEL, 1843) (ACTINOPTERYGII, CYPRINIDAE)

ABSTRACT

The genus *Luciobarbus* (Heckel, 1843) is among the most widespread and diverse primary freshwater fishes in the Palearctic region. This genus shows high levels of local endemism across its distribution range, with over 40 recognized species showing different habitat requirements. The genus has recently undergone taxonomic revisions with the incorporation of new species that have remained as synonyms until now. Previous studies concluded the separation within the group into two main lineages corresponding to Iberian and the rest of *Luciobarbus* species. In order to obtain a more thorough understanding of the relationships within the group, we enhanced sampling, including ~~five~~ 1 recently described species, constructed a phylogeny combining data from two *loci,* one mitochondrial gene (*cytb*) and one nuclear region (*actinII*) and assessed *Luciobarbus* biogeography across its known range. We support most of the current phylogenetic relationships and provide nuclear support for the three lineages in which is currently distributed the genus *Luciobarbus*.

Introduction

Among ray-finned fishes, Cypriniformes constitutes the largest and most diverse clade of freshwater fishes on the planet (Yang *et al.* 2015, Nelson *et al.* 2016). Within Cypriniformes, the subfamily Cyprininae account for 4% of bony fish diversity and is one of the most widespread subfamily in freshwater systems inhabiting southern Eurasia, Africa and America (Eschmeyer, 2015, Yang *et al.*, 2015). It is well-known that this subfamily counts with poliploid species resulting from genome duplications and hybridation phenomena during its evolution (Saitoh *et al.*, 2010). The classification of the subfamily Cyprininae has been subjected to debate in the last years (Yu *et al.*, 2000, Nelson, 2006, Wang *et al.* 2007, Saitoh *et al.*, 2006,Chen *et al.*2013, Yang *et al.*, 2010, 2012a, 2012b, 2012c, 2015). In polyploids, the different nuclear gene copies could substantially diverge and belong to different clades in a gene tree, which hampers the study of polyploid species (Evans *et al.*, 2005; Saitoh *et al.*, 2010, Yang et al, 2015).

Traditionally the tribe Barbini Bleeker, 1856 comprises the genera *Scaphiodonichthys, Semiplotus, Cyprinion, Aulopyge, Barbus, Luciobarbus* and *Capoeta*. Within this tribe, the genus *Capoeta* and *Luciobarbus* clustered together and are known to be evolutionary hexaploid and tetraploid, respectively (Machordom & Doadrio, 2001, Tsigenopoulous *et al.*, 2003, Banaraescu & Bogutskaya, 2003, Levin *et al.* 2012, Yang *et al.*, 2015).

The genus *Luciobarbus* Heckel, 1843 has a Palaeartic distribution, inhabiting the freshwater bodies of Asia, North Africa and southern Iberian Peninsula. The current systematic knowledge of *Luciobarbus* is based on several revisions of its morphology, osteology and molecular studies. Around 45 species within the genus *Luciobarbus* have been morphological and genetically described, so far (Geiger *et al.*, 2014, Casal-Lopez *et al.*, 2015, Doadrio *et al.*, 2016). These species are grouped in two major lineages

corresponding to Iberian Peninsula and other composed of the rest of *Luciobarbus*.

For Iberian *Luciobarbus* ~~and~~ Greek *L.albanicus* a different genus was proposed (*Messinibarbus,* Bianco, 1990) maintaining *Luciobarbus* for North African and Eastern Mediterranean species. The species *Luciobarbus subquincunciatus* has an ambiguous phylogenetic position with respect to both lineages and was not clustered neither in Iberian lineage nor in Eastern Mediterranean lineage, but showed a basal position to *Capoeta* (Levin *et al.*, 2012).

Overall, the Mediterranean region possesses a high number of endemic freshwater species (Abell *et al.*, 2008) and the genus *Luciobarbus* is one of the most diverse*. Luciobarbus* strictly occurs in freshwater habitats (though there is evidence of medium salt tolerance, Kraiem & Pattee, 1988) and can be found in large to small rivers of different typology. *Luciobarbus* is characterized by medium sizes species (up to 50 cm), showing a high degree of morphologial and genetic differentiation, with a body elongated, two pairs of barbels and a strong denticulated last single ray of the dorsal fin (Doadrio, 1990, Bănărescu & Bogutskaya, 2003)

Recently, ~~five~~ new species from Morocco have been described based on genetic and morphometric characters: *Luciobarbus rifensis* Doadrio, Casal-Lopez & Yahyaoui 2015, *Luciobarbus rabatensis* Doadrio, Perea & Yahyaoui 2015, *Luciobarbus maghrebensis* Doadrio, Perea & Yahyaoui 2015, *Luciobarbus guercifensis* Doadrio, Perea & Yahyaoui 2016, and *Luciobarbus zayanensis* Doadrio, Casal-López & Yahyaoui 2016.

The genus *Luciobarbus* have been the focus of many molecular studies in recent years due to its circum-Mediterranean distribution and Upper Miocene diversification in search of biogeographic clues in order to shed light on how this genus evolved during and after the Messinian Salinity Crisis. These phylogenies were once restricted to mitochondrial genes due to the difficulties of studying nuclear genes and/or due to incomplete sampling. This last also reflected in the fact that new species are still being described.

In this study we conducted ~~molecular~~ phylogenetic analyses using two ~~*loci*~~, one mitochondrial and one nuclear ~~marker,~~ coupled with a biogeographical reconstruction, to better depict phylogenetic relationships of *Luciobarbus* species and discuss the historical biogeography, especially on when and how they diversified.

Methods

Taxon sampling

Altogether, 35 different species of western Paleaeartic *Luciobarbus* are included in this study, with the exception of *Luciobarbus kottelati*, endemic from Menderes Basin in Turkey (Fig.1, Table.1 Supp). For phylogenetic purposes, *Barbus petenyi* included as outgroup based on previous phylogenetic (Markova *et al.* 2012). Fish were captured by electrofishing and fin clipped with the permission of the appropriate authorities. Fin clips were preserved in 96% ethanol and later stored at 4 °C. A few specimens from each sampling site were preserved in formalin as voucher specimens. Both DNA samples and specimens were deposited in the DNA and Fish Collection at the National Museum of Natural Sciences (MNCN-CSIC).

Fig.1. Map of distribution and sampling points of the species under study

DNA protocols and sequencing

Genomic DNA was extracted from ethanol-preserved tissue samples (fin clips) using DNA Blood&Tissue kit (QIAGEN). As the species of the genus *Luciobarbus* are known to be tetraploid (Machordom *et al.*, 1995, Berrebi *et al.* 1996), only a single nuclear gene, *actinII,* was found to be suitable for direct sequencing as described by Markova *et al.* 2010 ~~following the work of Atarhouch *et al.*, 2003~~. The complete mtDNA cytochrome *b* (*mt-cytb*; 1140pb) and a fragment of the intron 1 of nuclear gene actin (*n-actinII*; 489 bp) were amplified via polymerase chain reaction (PCR) of 100 and 93 individuals, respectively. Primers, amplification protocols and PCR products lengths for these *loci* are were as follows:

Table1. Primer and PCR protocols used

Gene	**Primer name**	**Sequence 5'-3'**	**Tann (C^0)**	**Source**
cytb	GludGL H16460	TGACTTGAAR AACCA YCGTGG CGAYC TTCGG ATTAA CAAGA CCG	48	(Palumbi,1996) (Doadrio & Perdices, 2001)
actin-II				(Markova *et al*, 2010)

In all cases, PCR mixtures were prepared under similar conditions in a final volume of 11 µl containing 1-1.5 µl DNA, 0.25 µM each primer, 0.25 mM each dNTP, and 0.15 µl of Taq DNA polymerase (VWR). After checking PCR products on 1.5% agarose gels, the four genetic fragments were purified with the kit ExoSAP-IT™ (USB) and directly sequenced on MACROGEN Inc. (Seoul, Korea; http://www.macrogen.com).

Sequences were aligned using the default pairwise and multiple alignment parameters in Clustal W (Thompson *et al.*, 1994) implemented in MEGA software v.6.0 (Tamura *et al.*, 2013). Nuclear data was unphased with the PHASE algorithm in DNAsp v.5.0 (Librado and Rozas, 2009). Recombination of the *n-actinII* gene (p = 0.46) was tested using the PHI test in Splitstree v. 4.13 (Huson and Bryant, 2006).

Phylogenetic analyses

The best-fit model of molecular evolution for each gene and codon position was estimated via AIC in PartitionFinder v. 1.1.1 (Lanfear *et al.*, 2012). Best partition schemes used in different phylogenetic analyses based on the selected evolutionary models were:

Gene	**Partition I**	**Partition II**	**Partition III**
cytb	K80+I+G	TrN+I	TrN+I+G
actinII	HKY+I	HKY+I	HKY+I

Bayesian inference (BI) was performed using MrBayes v.3.2 (Ronquist *et al.*, 2012). Two simultaneous analyses were run for 10^7generations, each with four MCMC chains sampling every 100 generations. After discarding the first 10% of generations as burn-in, the 50% majority rule consensus tree and posterior probabilities were obtained. Phylogenetic analyses of

both genes, independently and concatenated, were conducted using Maximum Likelihood (ML) in RaxML software implemented in the Trex-online server (Stamakis, 2006) using the substitution model GTRGAMMA and the rapid bootstrap algorithm. Node confidence in ML analyses was estimated by rapid bootstrapping using a random seed (1000 replicates).

Estimation of the ages of divergence and diversification

Divergence times and their credibility intervals (highest posterior density: HPD) were estimated using BEAST v.1.7 software (Drummond and Rambaut, 2007) under a Bayesian framework. We implemented a single lognormal relaxed clock model under a Birth-Death model and constrained the age of four key nodes in accordance with the fossil evidence for *Luciobarbus*: *Luciobarbus sp*. from Pasalar (Turkey) dated as 14-15 mya (http://www.wahre-staerke.com/): *Luciobarbus subtruncatus (*Heckel, 1843) from Vösendorf, Austria, dated ~10.5 Ma (Boheme, 2002) *Luciobarbus sp*. from Iberian Peninsula dated around 6 mya (Doadrio & Casado, 1989). The fourth node, dated around 5.3 mya was based on the ~~study of Machordom and Doadrio, 2001.~~

We ran the BEAST analysis three times with random starting trees in order to ensure convergence on the global optimum. MCMC analyses were run for 50 million generations, with parameters logged every 10 000 generations. The remaining parameters were used as default. Output from BEAST was examined in Tracer 1.6 (http://tree.bio.ed.ac.uk/software/tracer/) to ensure convergence and ESSs >200, and the results were summarized using TreeAnnotator 1.7 (Drummond *et al.*, 2012).

We used a lineage-through-time (LTT) approach to assess changes in diversification rates throughout evolution of the groups, (Harvey *et al.*, 1994; Nee *et al.*, 1994; Barracough & Nee, 2001). We used the R library APE (Paradis *et al.*, 2004), using the mt-cytb and n-actinII ultrametric trees obtained in BEAST. Lineage-through-time plots represent the time elapsed

between successive branching events (Barraclough & Nee, 2001). The ultrametric tree contains information on the number of lineages and the molecular distance of every lineage from the root (the relative time of each node from the root node). We used the γ-statistic (Pybus & Harvey, 2000) to test for temporal shifts in diversification rate.

Ancestral area reconstruction

We reconstructed the ancestral states of the distribution ranges of the genus *Luciobarbus* using the Dispersal–Extinction–Cladogenesis (DEC) model of LAGRANGE (Ree and Smith, 2008) implemented in RASP v.3.1 software (Yu *et al.*, 2015). The ultrametric and dichotomous tree obtained for the *mt-cytb* gene in BEAST analysis was used as the tree topology on which mapping ancestral areas. Tips of the ultrametric tree were code by areas in order to reconstruct ancestral states.

Results

Phylogenetic analyses and divergence estimation

Based on the mitochondrial marker *cytb* the phylogenetic analysis supported the existence of two lineages, one comprised by *L. mursa*, which is distributed in tributaries of Caspian Basin and two lakes from Iran; and another one comprised by the rest of the *Luciobarbus* species. These two lineages will be called lineage A and B, respectively from now on. Based on our divergence estimation, the split between lineage A and B took place around 13.6 ~~mya~~ (11.8-16.2 ~~Mya~~ in 95% HPD), falling in the Middle Miocene (Fig. 2 and 3).

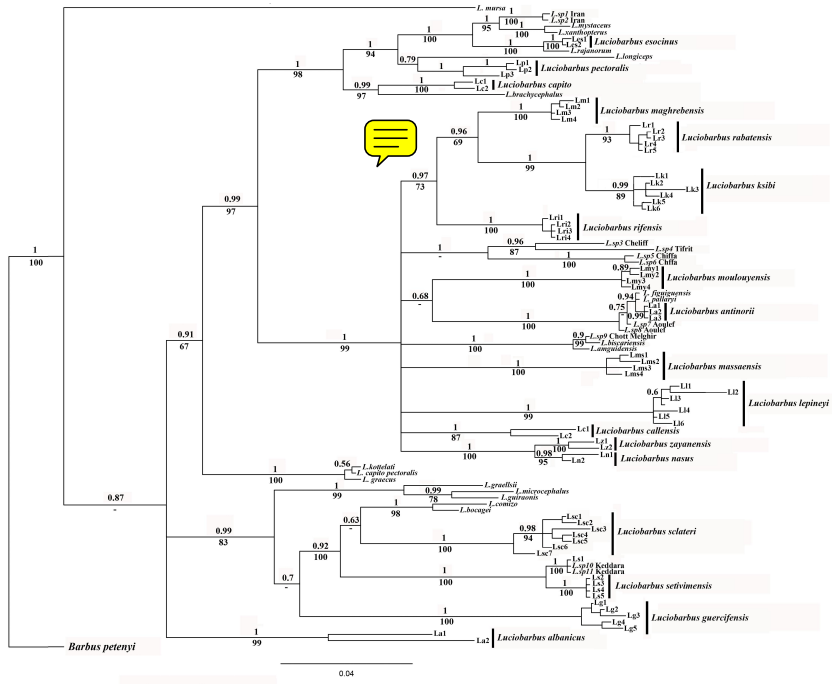

Fig.2. Phylogenetic tree of *cytb* gene rendered by Bayesian Inference (BI). Numbers on branches indicate posterior probability values for BI and under branches indicate bootstrap for ML.

Within lineage B we found three distinct clades: Clade I comprised two individuals of *Luciobarbus albanicus* from Greece, which show a deep divergence between them and that split from the rest around 9.5 mya (7.4-11.6 mya in 95% HPD). Clade II, estimated to have been separated from the rest around 6.5 mya (5.7-7.4 mya in 95% HPD), was composed of the Iberian species and two North African taxa, *L.setivimensis* from Algeria and *L. guercifensis* from Morocco. In the case of *L.setivimensis* we found that populations are geographically structured. Clade III included the Eastern and the rest of North African species of *Luciobarbus,* which were estimated to split from the rest around 9.~~03~~ mya (7.4-10.7 mya in 95% HPD).

The relationships between these three clades were unresolved and the divergence between them was estimated to have occurred during upper-Miocene, around 11 mya (9.3-13.1 in 95% HPD).

Within clade II, the Iberian species are not monophiletic, since *L.setivimensis* from Algeria is sister group to Iberian *L.sclateri, L.comizo, L.bocagei* with respect to the rest of Iberian species *L.guiraonis, L.graelsii* and *L.microcephalus.* The position of North African species *L.guercifensis* was not clearly resolved due to a poor support.

Within this clade a first split between Moroccan *L.guercifensis* and the rest took placed around 6.5 Mya (5.7-7.2 in 95% HPD). Subsequently, the Iberian species *L.microcephalus, L.guiraonis* and *L.graellsii* split from the rest around 6.2~~4~~ Mya (5.5-7.2 in 95% HPD), followed by two splits: a first one between Iberian *L.sclateri* and the rest around 5.3 Mya (5.4-5.2 in 95%HPD) and a second one, between Algerian *L.setivimensis* and *L.comizo* and *L.bocagei* around 4.9 Mya (3.9-5.3 Mya in 95% HPD).

Within clade III, we found two distinct subclades: subclade A, comprised by species from Aegean Basin in Turkey and Greece: *L.lydianus* and *L.graecus;* and subclade B, composed by the rest of *Luciobarbus* species. The divergence between subclade A and B was estimated around 10.2 mya (8.7-11.8 mya in 95% HPD).

Within subclade B, we found two monophyletic groups: a first one formed by North African species in which some relations were recovered but in general showing poorly resolved phylogenetic relationships; and a second group, formed by Eastern species that show well resolved relationships. The split between these two groups occurred around 9~~.03~~ Mya (7.4-10.7 Mya in 95% HPD).

In the group of North African species we recovered the relationships of the species present in the north-western Morocco, *L. maghrebensis L. rabatensis* and *L. rifensis* and *L.ksibi. L.rifensis*, ~~geographically close to the Iberian Peninsula,~~ was basal species with respect to the others. This group was estimated to have diverged from the rest around 5.1 mya (2.7-5.26 mya in 95% HPD).

The Saharian species were group into three clusters: the first one formed by *L.lepineyii* from west Morocco, which split from the rest around 6.69 mya (5.19-8.26 mya in 95% HPD); the second one comprised by eastern Algerian species *L.amguidensis, L.biscariensis* and *L.sp* from Chott Melghir, that split around 4.8 mya (3.4-6.15 mya in 95% HPD); and a third one formed by *L.figuiguensis, L.pallaryi* and *Aoulef* populations from western Algeria, and also western *L.antinorii*, which come from Chott el Djerid in Tunisia, ~~which was a surprising discovery.~~ Rheophilic species, *L.zayanensis* and *L.nasus*, from Oum er Rbia and Tensif basins were clustered together. As we have previously introduced, another rheophilic species, *L.guercifensis* was nonetheless included in the Iberian clade. We also found a series of populations from Mediterranean rivers from Algeria which could not ~~be morphological nor genetically~~ recognized species.

The divergence between North African species seems to have occurred between the upper Miocene and Pliocene. The molecular clock found that *L.lepineyi* was the first species to diverge, around 6.6 mya (5.19-8.26 mya in 95% HPD).

Within Eastern species, the relationships are well supported. An initial split was estimated to have occurred around 5.~~28~~ mya (3.8-7.01 mya in 95% HPD) in which the Caspian species, *L.capito* and *L.brachycephalus* are positioned as sister group of the rest. Syrian species *L.longiceps* and *L.pectoralis* from Mediterranean basins in the Near East were clustered together with respect to *L.esocinus, L. xantoptherus, L.mystaceus* from the Caspian Basin, Tigris-Euphrates and Persian Gulf basins. We found two individuals from Iran that are not yet assigned to any recognized species.

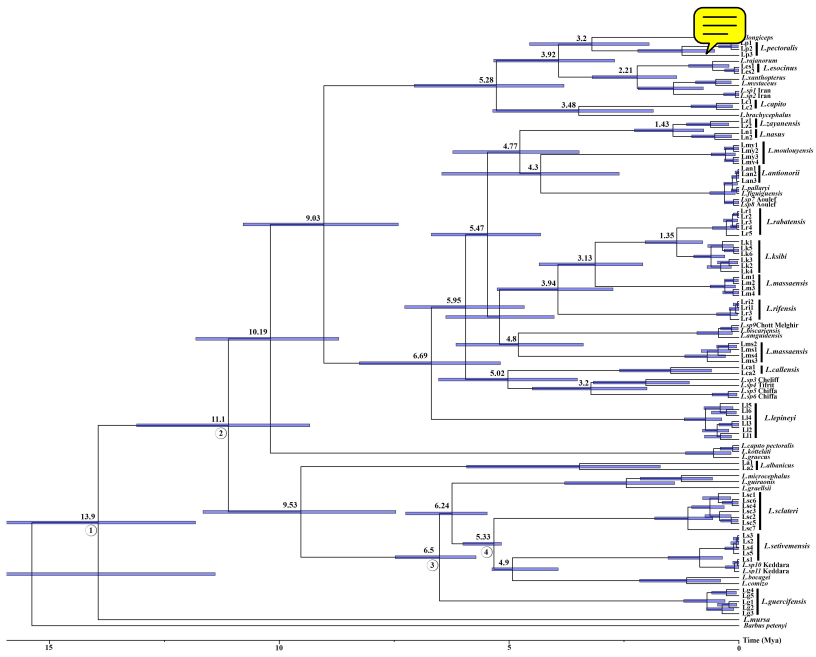

Fig 3.- Calibration tree and divergence times based on *cytb* gene and estimated through fossil record for the species of the genus *Luciobarbus*. Numbers, above branches represent divergence ages estimation and the blue bar represent their HPD 95% confidence intervals, respectively.

In the nuclear phylogeny the relationships were poorly resolved. Discrepancies were found in the position of *L. setivimensis*, which is clustered with geographically close North African species rather than with the Iberian in *n-actinII* phylogeny. Iberian species showed low values of support, but the inclusion of North African *L. guercifensis* should be noted, since this relationship was also found and in *mt-cytb* phylogeny strongly supported. Some alleles are shared between species in the case of some north African species (Fig4 and Fig 5).

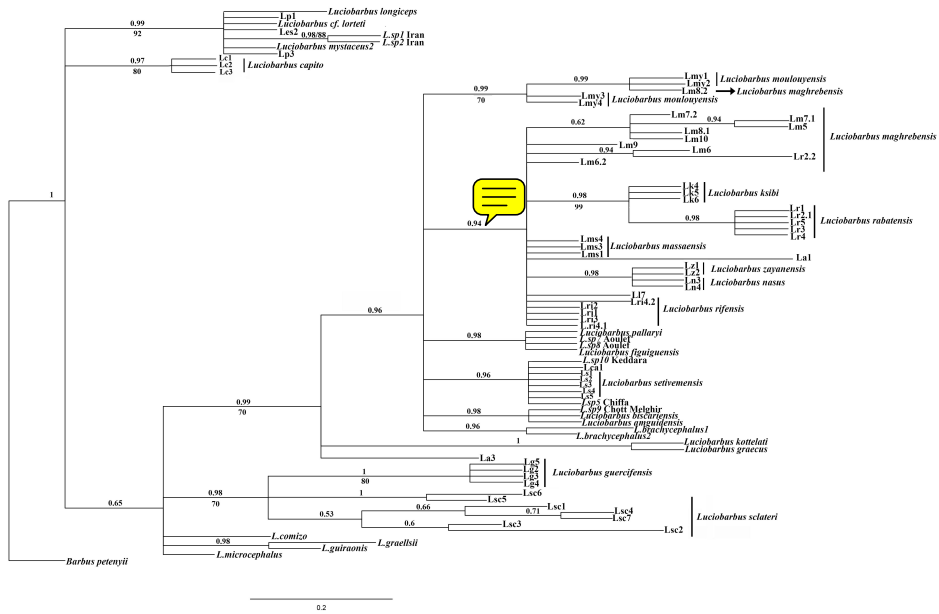

Fig.4. Phylogenetic tree of *actinII* gene rendered by Bayesian Inference (BI). Numbers on branches indicate posterior probability values for BI and under branches indicate bootstrap for ML.

Biogeographical reconstruction and rate of diversification

The ancestral area reconstruction using DEC method show a biogeographical history followed by *Luciobarbus* in which dispersal and vicariant events were frequent. Overall, 16 dispersal and 9 vicariance events were detected. North Africa was the region with the highest number of speciations within areas, counting with 65 speciation processes, followed by Iberian Peninsula with 11 and Tigris-Eufrates Basin with 4 (Fig.7). We found that in *Luciobarbus* events such as dispersion and vicariances occurred from upper Miocene to the end of Pliocene. In the Lago Mare Phase, which took place around 6 mya, we found that the occurrence of dispersion was only detected between geographically close areas from Iberian Peninsula and North Africa. In contrast and during this period, we did not detect dispersions that may explain biogeographical relations between long-distance areas of the Mediterranean Basin. Dispersion between big biogeographical areas seems to have occurred in upper-mid Miocene before the Messinian period. Some of the vicariant processes detected are highly

relevant for the present-day dispersion patterns of some of the extant groups of species, such as the one occurring between *L.lydianius* and *L.graecus*, species located to each sides of the Aegean Basin, one in Turkey and the other in Greece. Moreover, another example of important vicariance event occurs between *L.xanthopteurs*, *L.mystaecus* and the species from Iran, that coincides with the separation of the Tigris-Euphrates and Persian Gulf basins. Both events, seem to have occurred close to the Pleistocene period. Our results also shows another vicariant event which seem to have occurred during the Messinian Salinity Crisis, in which the Caspian Basin seem to have split from the rest of the Eastern Basins.

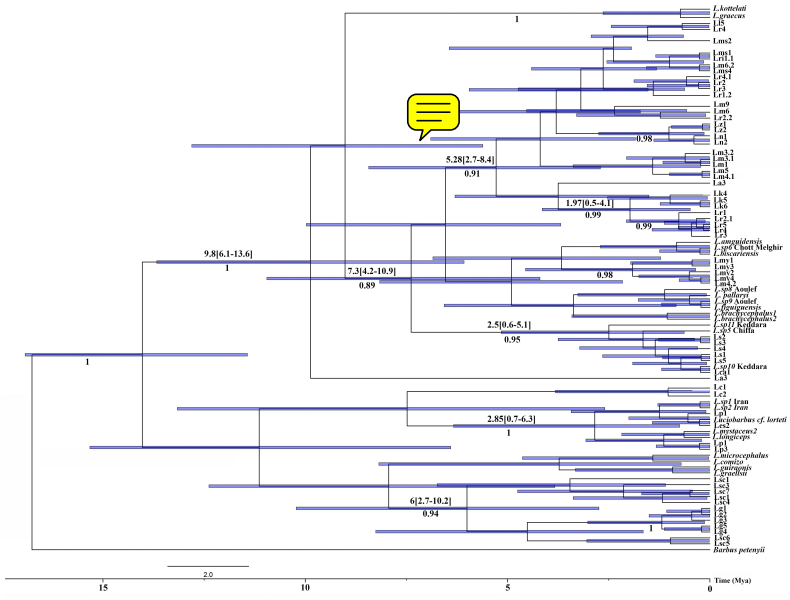

Fig 5.- ~~Calibration~~ tree and divergence times based on *actinII* gene and estimated through fossil record for the species of the genus *Luciobarbus*. Numbers, above branches represent divergence ages estimation and the blue bar represent their HPD 95% confidence intervals, respectively.

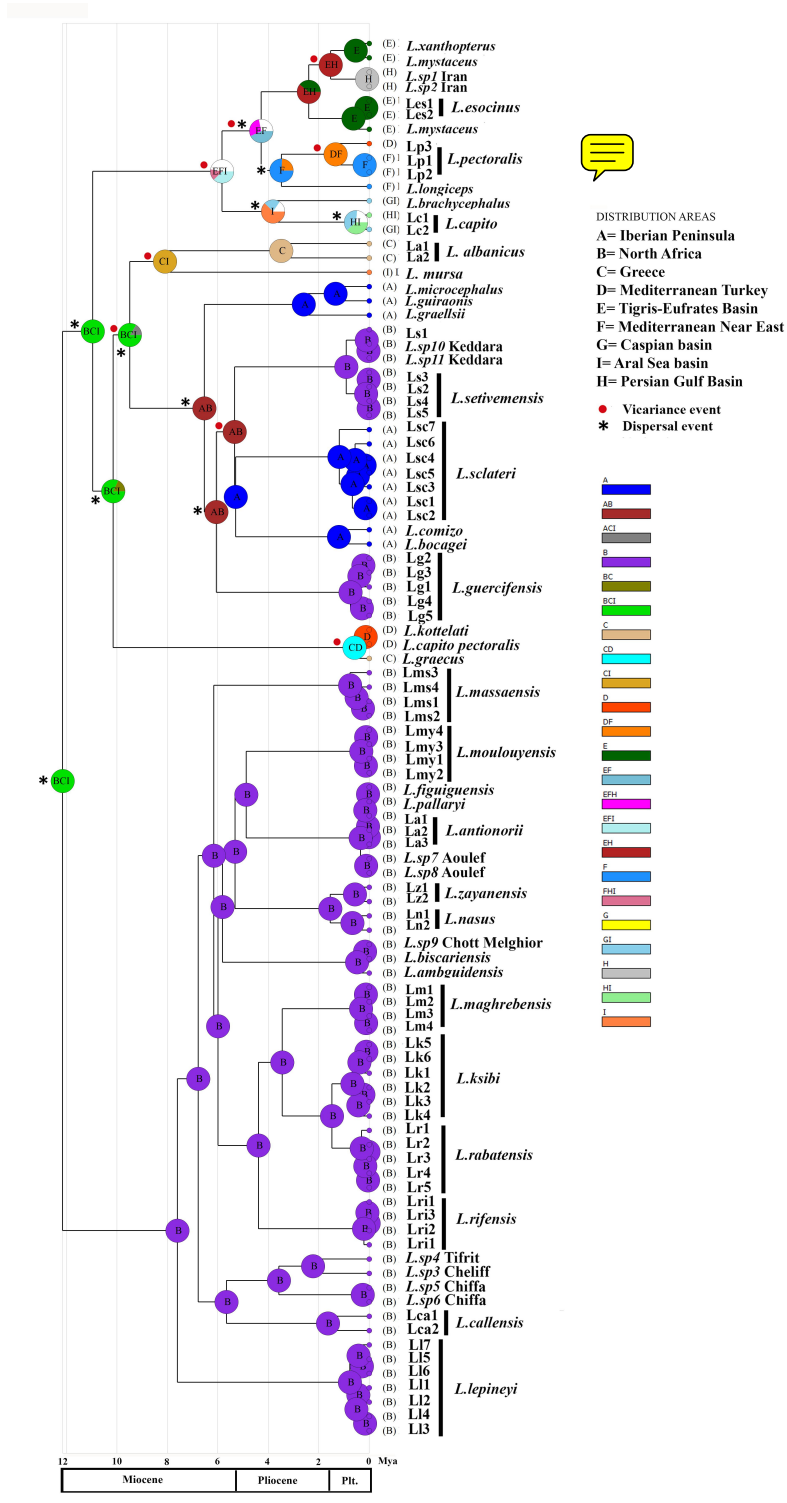
(E) L.xanthopterus
(E) L.mystaceus
(H) L.sp1 Iran
(H) L.sp2 Iran
Les1 L.esocinus
Les2
(E) L.mystaceus
Lp3
Lp1 L.pectoralis
Lp2
(F) L.longiceps
(GI) L.brachycephalus
Lc1
Lc2 L.capito
La1
La2 L. albanicus
(I) L. mursa
(A) L.microcephalus
(A) L.guiraonis
(A) L.graellsii
Ls1
L.sp10 Keddara
L.sp11 Keddara
Ls3
Ls2
Ls4
Ls5 L.setivemensis
Lsc7
Lsc6
Lsc4
Lsc5
Lsc3 L.sclateri
Lsc1
Lsc2
L.comizo
L.bocagei
Lg2
Lg3
Lg1 L.guercifensis
Lg4
Lg5
L.kottelati
L.capito pectoralis
L.graecus
Lms3
Lms4 L.massaensis
Lms1
Lms2
Lmy4
Lmy3
Lmy1 L.moulouyensis
Lmy2
L.figuiguensis
L.pallaryi
La1
La2 L.antionorii
La3
L.sp7 Aoulef
L.sp8 Aoulef
Lz1
Lz2 L.zayanensis
Ln1
Ln2 L.nasus
L.sp9 Chott Melghior
L.biscariensis
L.ambguidensis
Lm1
Lm2
Lm3 L.maghrebensis
Lm4
Lk5
Lk6
Lk1
Lk2
Lk3 L.ksibi
Lk4
Lr1
Lr2
Lr3 L.rabatensis
Lr4
Lr5
Lri1
Lri3
Lri2 L.rifensis
Lri1
L.sp4 Tifrit
L.sp3 Cheliff
L.sp5 Chiffa
L.sp6 Chiffa
Lca1
Lca2 L.callensis
Ll7
Ll5
Ll6
Ll1 L.lepineyi
Ll2
Ll4
Ll3
12 10 8 6 4 2 0 Mya
Miocene
Pliocene
Plt.
DISTRIBUTION AREAS
A= Iberian Peninsula
B= North Africa
C= Greece
D= Mediterranean Turkey
E= Tigris-Eufrates Basin
F= Mediterranean Near East
G= Caspian basin
I= Aral Sea basin
H= Persian Gulf Basin
Vicariance event
Dispersal event
A
AB
ACI
B
BC
BCI
C
CD
CI
D
DF
E
EF
EFH
EFI
EH
F
FHI
G
GI
H
HI
I

Figure 6. Ancestral area reconstruction of *Luciobarbus* populations on a *cytb* tree generated by BEAST using Dispersal-Extinction-Cladogenesis (LAGRANGE) model implemented in RASP software. Legend of colours represents possible ancestral ranges. The Most Likely State is represented at the pie chart, with the code and colour corresponding to the area it is assigned.

The LTT plot (Fig.7), reflecting the temporal pattern of diversification, showed an exponential increase in lineages that is intensified at the end coinciding with the Plio-Pleistocene, when most of the current species appeared.

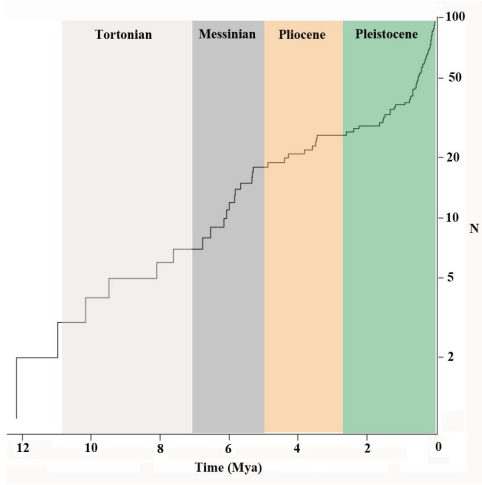

Figure 7. Lineage through time plot (LTT) obtained from the ultrametric tree in Fig. 6.

Discussion

From Oligocene times onwards the Mediterranean region underwent a dynamic orogenic and tectonic activity with the formation of Mediterranean mountain chains and two most important residual oceans, the Paratethys to the north and the Mediterranean to the south (Seneš, 1973 and Steininger and Wessely, 1999, Popov *et al.* 2006). Thus, this area represents an intricate scenario to carry out diversification studies. This complex

geological history is reflected in the existence of a large number of endemic species in relatively small areas along the Mediterranean region. This pattern is known for many other groups with Mediterranean distribution (Myers et al, 2000, Hrbek and Meyer, 2002, Machordom and Doadrio, 2001, Doadrio and Perdices, 2005, Murienne et al, 2010).

Luciobarbus phylogeny

Our phylogeny resolved some of the basal nodes that were not yet resolved in previous works, and that in some cases have been interpreted as a rapid radiation through Lago Mare Phase in the Mediterranean (Tsinegopoulos *et al.*, 2007). In this sense, the basal politomy of the *Luciobarbus* relations was not resolved until this paper (Geiger *et al.*, 2014, Levin *et al.*, 2012, Tsinegopoulous *et al.* 2007).

We found that the first split within the genus *Luciobarbus* which separated *L. mursa* from the rest probably happened in the Middle Miocene when the eastern Paratethys began to be isolated from the central Paratethys (Ter Borgh *et al.*, 2014). This division into two different areas due to the extension of the Carpathian Foreland occurred in this period and has been also claimed for other strictly freshwater organisms (García-Vázquez *et al.*, 2016). A first split could have taken place in the drainages flowing into the Caspian Sea, in the light of the distribution of *Luciobarbus mursa*, a species restricted mainly to this area.

Our phylogenetic results show a second split that gave rise to three clades: Clade I, *L. albanicus;* Clade II, mainly Iberian species; and clade III, Eastern and most of North African species.

The present distribution of clade III is strongly supported by a series of palaeogeographical events occurring during the Miocene. During late middle Miocene, sea regression in the Arabian Plateau ~~(Meulenkamp *et al.*, 2003)~~, allowed connection with Africa, ~~which~~ may have opened a route of dispersion for *Luciobarbus* from Middle East to Africa as predict in our

DEC analysis. This continental route of connection between Eurasia and Africa has been also postulate for other cyprinids (Otero *et al.*, 2001,Perea *et al.*, 2010). The posterior reopening of the Red Sea channel during the upper Miocene (Meulenkamp *et al.*, 2003) should have caused the isolation of North African populations, in agreement with our divergence estimation. This scenario was already proposed by another author (Doadrio 1990). Nonetheless, this scenario is contrary to the classical theories in which the dispersions were produced from Iberian Peninsula to North Africa (Banarescu 1960; Almaça 1976, 1988; Banarescu 1989, 1992).

On the other hand, the current distribution of clade I and clade II is more uncertain based on palaeogeographic data. Dispersion towards west Europe and eastern Greece could be related to the formation of the Pannonian Basin as consequences of the uplift of the Eastern Alps and substantial microplate rotations and basin inversion in the Pannonian-Carpathian-Dinaric realm during the middle Miocene (Paramova *et al.* 2004, Vrabec and Fodor, 2006, Kuhlemman *et al.*, 2007, Hinsbergen *et al.*, 2008). This Pannonian basin was isolated from marine basins that resulted in shifts of salinity, due to an increase of freshwater input. This caused the freshening of water conditions and thus, the extinction of marine lineages in Central Paratethys and the evolution of endemic brackish-water lineages (Magyar *et al.*, 1999, Harzhauser and Tempfer, 2004, Popov *et al.*, 2004, Krijgsman *et al.* 2010). Eventually a permanent lake was formed, the Lake Pannon that existed from 12 to 4 mya (Magyar *et al.* 1999, Sacchi and Horvat, 2002, Grigorovich *et al.,* 2003, Vasiliev *et al.,* 2010 and Ter Borgh *et al.*, 2013, 2014). This will be in agreement with the absence of fossils and the absence of present-day species of the genus *Luciobarbus* in the area within the Balkans, due to the impossibility of colonization through Pannonian Basins.

Although nowadays there are no *Luciobarbus* in central Europe, the presence of *Luciobarbus* fossils from the Pannonian at Vösendorf (Vienna Basin) dated to 10.5 Ma proved the presence of *Luciobarbus* once in this

area (Böhme, 2002). So, the absence of extant species of the genus *Luciobarbus* in the freshwater systems of central Europe may be due to a posterior extinction of the populations mediated by environmental and climatic shifts that happened in posterior periods (Böhme 2003, 2010, 2014). For another freshwater fish *Cottus gobio* suggested a Paratethyan origin. It would have invaded central Europe via the ancient Danube and posteriorly disperse to the Mediterranean France through connection of the upper Danube, upper Rhine and Rhône rivers (Enelbrecht *et al.*, 2000). Nonetheless, this route does not seem plausible for *Luciobarbus.* Whereas, *Cottus gobio* is an alpine fish that can live in fast currents and cold waters (Freyhoff & Kottelat), *Luciobarbus* species have different ecological requirements, with preference for ~~temperate~~ waters and middle-lower courses of rivers (Kottelat & Freyhof, 2007, Casal-López *et al.*, 2015, Doadrio *et al.*, 2016). The ways and modes by which *Luciobarbus* colonized the Iberian Peninsula form Europe still remains an enigma.

The presence of *L. albanicus* in Greece could be explained by the existence of the Vardar thrust that could have led to connection with the Pannonian Lake (Zelic *et al.*, 2010). The presence of *L albanicus* in this area has not been previously registered and the presence of *Luciobarbus* in the Iberian Peninsula has been postulated through continental bridges or through the Lago Mare Phase. Nonetheless, none of these explanations seem to fit our phylogenetic and biogeographic results.

Within clade II was conformed mainly by Iberian species but two species currently distributed for ~~s~~ort-Africa appear in this clade: The clustering of *L.setivimensis* with the Iberian species has already been observed in previous studies (Machordom *et al.*, 1998, Machodrdom and Doadrio, 2000, Levin *et al.*, 2012). The present-day distribution of *L. guercifensis* and *L. setivimensis,* phylogenetically close to the Iberian species, in the eastern region of the Gibraltar Arc, could be related to the rapid block rotation of this system (Crespo-Blanc, 2016). Indeed, the biogeographical reconstruction supports a dispersal-vicariant event for explaining the

current distribution of these species. Nonetheless, there is a phylogenetic discordance in the position of *L. setivimensis*: In contrast to mitochondrial results, the nuclear data place *L. setivimensis* with the North African and not with the Iberian species. We postulate that the presence of the nuclear genome of *L.setivimensis* in the Setif and Soumman basins is due to later secondary contact, while the mitochondrial genome remained from the earlier state. This is a common finding in European cyprinids, in which the partial or complete presence of mtDNA of different species occurs (Durand *et al,* 2002, Perea *et al.*, 2016).

I n clade III we recovered the same phylogenetic relationships that those found by others authors (Zardoya & Doadrio 1990, Levin *et al.*, 2012, Geiger *et al.*, 2014), in which North African and Eastern species are sister group to *L. graecus* and *L. lydianus* from Greece and Turkey, respectively.

Overall, within the North African sublcade, the relationships were poorly resolved. The western species *L. rabatensis*, *L. rifensis*, and *L. maghrebensis* are grouped together with *L. ksibi*, being *L. rifensis* as basal to the rest. A previous work recovered the relation between these species, although in that work they were not yet assigned to any species (Geiger *et al.*, 2014).

The Saharian species were clustered in three *mt-cytb* groups corresponding to: group A, *L. lepineyi* from Dra and Noun basins in western Sahara; group B, *L. biscariensis* from Biskra (eastern Algeria) and *L. amguidensis* from Imirhou (south eastern Algeria) basins, and a population from Chott Melghir, in eastern Algeria; group C, *L. pallaryi* and *L. figuiguensis* from Zousfana Basin, in southern Morocco, with *L. antinorii* from Chott el Djerid in western Tunisia. These phylogenetic relations were recovered in previous works with allozymes, *mt-cytb* and *mt-coxI* (Machordom *et al.*1998, Machordom and Doadrio, 2001, Geiger *et al.*, 2014) and in this study we have also recovered them with *n-actinII*. Due to the fact that *L. antinorii* is probably an extinct species, its phylogenetic relationships

were only studied through allozymes and *cytb*. Surprisingly, in both cases, this species was phylogenetically closer to the western Saharan populations than to eastern populations (Machordom *et al.*, 1998, Machordom and Doadrio, 2001).

Currently, Dra and Noun basins, where *L. lepineyii* is distributed flow into the Atlantic Ocean. The Zousfana basin, where *L. pallaryi* and *L. figuiguensis* are present flows to an endhorreic basin Sebkha el Melah. A geographical grouping could be found for these species, but *L. antinorii* inhabits Chott el Djerid, one of the major discharge areas from the Complexe Terminal aquifer (Roberts and Mitchell, 1987).

The northern Sahara contains two important aquifer systems: the Continental Intercalaire (CI) overlain by the Complexe Terminal (CT). The CI is a hydraulically continuous aquifer that has its source in the Atlas Mountains in Algeria and discharges in the Chotts of Tunisia and in the Gabès golf (Edmund *et al.*, 2003). Its waters are related to a palaeoclimatic recharge, which occurred during the Late Pleistocene and the Early Holocene humid periods (Hamed *et al.* 2013). By contrast, the CT is unconfined or semi-confined, with its main recharge area in the central Sahara. The so-called zone of Chotts (Chott El Jerid, Chott El Gharsa and chott Melrhir) is characterized for a complex geological past (Aloui *et al.*, 2012). At western parts of the Chotts the development of floodplain areas during Miocene could be more frequent (Aloui *et al.* 2012). This could lead to contact within adjacent fluvial networks and could explain some of the phylogenetic relationships that we found such as the one between *L. biscariensis* and Chott Melghir population.

Within the Eastern subclade, we found a vicariant event at the end of the Miocene which divided the species present in Aral Sea, *l. brachycephalus and capito* from the rest. This is in agreement with the isolation of the Caspian Sea since late Miocene. From late Miocene to the

Pliocene, the basins of the Caspain Sea and Black Sea became separated (Grigorovich *et al.*, 2003).

The separation of the *Luciobarbus* species of the Tigris and Euphrates and Persian Gulf (*L. xanthopterus, L. esocinus, L. mystaceus, L.sp1* and *L.sp2* from Iran*)* from the rest of species form Mediterranean Turkey and Mediterranean Near East would coincides with the formation in the Pliocene of the Tigris Euphrates basins when the water from the developing Zagros Mountains drained (Banister, 1980). The subdivision of the species from Tigris-Euphrates from the species of *Luciobarbus* from Persian Gulf falls in Late Pliocene. This scenario fits the vicariant and dispersion events that our DEC analysis found for explaining the patterns of distribution of eastern *Luciobarbus* species.

CONCLUDING REMARKS

To sum up, the phylogenetic organization of *Luciobarbus* is composed of two main lineages with *L. mursa* as first species to diverge. The end of the Miocene (10-6 mya), was a period especially important for the diversification of the genus *Luciobarbus* into three main clade: the Greek *L. albanicus,* the Iberian clade (in which Moroccan *L. guercifensis* and Algerian *L. setivimensis* are included), and the Easter and North African group. During this period several important changes occurred in the Mediterranean the fragmentation of Parathetys in different basins and the isolation of the Mediterranean basin and the connection between The Arabian Plateau and North Africa. According to our molecular clock, the history of the genus *Luciobarbus* depicts a Middle Miocene boundary in which Middle East appears as the origin of the current distribution of *Luciobarbus* in western Mediterranean. Currently, these species constitute endemism of their distribution areas in which allopatric speciation processes through currently and historical freshwater networks isolated and connected across the Mediterranean.

Supplementary material

Table 1S. Species and outgroup within the genus *Luciobarbus* included in phylogenetic analysis and molecular clock.

Current species assigantion	Freshwater system	Country	N	Code in tree
L.sp1	Ghareh Aghaj River	Iran	1	*L.sp1* Iran
L.sp2	Zakhem River	Iran	1	*L.sp2* Iran
Luciobarbus mystaceus1	Merziman Stream	Turkey	1	*L.mystaceus1*
Luciobarbus mystaceus2	Dicle 5km west of Hasankeyf	Turkey	1	*L.mystaceus2*
Luciobarbus xanthopterus	Tigris River	Turkey	1	*L.xanthopterus*
Luciobarbus esocinus	Tigris River	Turkey	1	Les1
Luciobarbus esocinus	Dicle 5 km west of Hasankeyf	Turkey	1	Les2
Luciobarbus mystaceus	Keban Dam Lake (Euphrates R)	Turkey	1	*L.mystaceus3*
Luciobarbus longiceps	Yarmuk River	Syria	1	*L.longiceps*
Luciobarbus pectoralis	Asi River	Syria	1	Lp1
Luciobarbus pectoralis	Qattinah Lake	Syria	1	Lp2
	Ceyhan River	Turkey	1	Lp3
	Goksu River	Turkey	1	Lp4
Luciobarbus capito	Tejan River	Iran	1	Lc1
Luciobarbus capito	Terek River	Rusia	1	Lc2
Luciobarbus brachycephalus	Terek River	Rusia	1	*L.brachycephalus*
Luciobarbus maghrebensis	Ifrane River	Morocco	4	Lm1-Lm4
Luciobarbus rabatensis	Grou River		5	Lr1-Lr5
Luciobarbus Ksibi	Reraia	Morocco	2	Lk1-Lk2
Luciobarbus ksibi	Kasab	Morocco	2	Lk3-Lk4
Luciobarus ksibi	Oum er Rbia	Morocco	2	Lk5-Lk6
Luciobarbus rifensis	Hachef	Morocco	3	Lri2-3
Luciobarbus rifensis	Laou	Morocco	1	Lri1
L.sp3	Cheliff	Algeria	1	*L.sp3* Cheliff
L.sp4	Tifrit	Algeria	1	*L.sp4* Tifrit
L.sp5	Chiffa River	Algeria	1	*L.sp5* Chiffa
L.sp6	Chiffa River	Algeria	1	*L.sp6* Chiffa
Luciobarbus ~~moulouyensis~~	Zobzite River	Morocco	3	Lmy1-Lmy3-Lmy4
Luciobarbus ~~moulouyensis~~	Moulouya River	Morocco	1	Lm2
Luciobarbus figuiguensis	Ain Dzadert River	Morocco	1	*L.figuiguensis*
Luciobarbus payarii	Guir River	Morocco	1	*L.payarii*
Luciobarbus antinorii	Bichri River	Tunisia	3	La1-La3
L.sp7	Aoulef	Algeria	2	*L.sp7* Aoulef
L.sp8				*L.sp8* Aoulef
L.sp9	Chott Melghir	Algeria	1	*L.sp9* Chott Melghir
Luciobarbus biscariensis	El Abiod	Algeria	1	*L.biscariensis*
Luciobarbus amguidensis	Imirhou	Algeria	1	*L.amguidensis*
Luciobarbus massaensis	Tamrhalcht	Morocco	1	Lms1
	-		-	Lms2
	Assaka		1	Lms3
	Zag Mouzen		1	Lms4

Luciobarbus lepineyi	Tidil	Morocco	1	Ll1
	Noun		1	Ll2
	El Maleh		1	Ll3
	Bouia		1	Ll4
	Dra		2	Ll5, Ll7
	Ziz		1	Ll6
Luciobarbus callensis	Kebir	Algeria	2	Lc1, Lc2
Luciobarbus zayanensis	Nfiss, Douar Imaounane	Morocco	1	Lz1
	Nfiss, Ouirgane		1	Lz2
Luciobarbus nasus	Serrou	Morocco	1	Ln1
	Oum er Rbia		1	Ln2
Luciobarbus kottelati	Hamam stream	Turkey	1	*L.kottelati*
Luciobarbus capito pectoralis	Tarsus River	Turkey	1	*L.capito pectoralis*
Luciobarbus graecus	Kifissos	Greece	1	*L.graecus*
Luciobarbus graellsii	Arga	Spain	1	*L.graellsii*
Luciobarbus microcephalus	Zújar	Spain	1	*L.microcephalus*
Luciobarbus guiraonis	Mijares	Spain	1	*L.guiraonis*
Luciobarbus comizo	Guadalmez	Spain	1	*L.comizo*
Luciobarbus bocagei	Ibor	Spain	1	*L.bocagei*
Luciobarbus sclateri	Calaboza	Spain	1	Lsc1
	Posadas	and	1	Lsc2
	Manilva	Portugal	1	Lsc3
	Guadalmena		1	Lsc4
	Jándula		1	Lsc5
	Odiel		1	Lsc6
	Segura		1	Lsc7
Luciobarbus setivimensis	Aissi	Algeria	1	Ls1
	Soumman		1	Ls2-Ls5
L.sp10	Keddara	Algeria	2	*L.sp10*
L.sp11				*L.sp11*
Luciobarbus guercifensis	Melloulou	Morocco	1	Lg1
	Moulouoya		3	Lg2-Lg3
Luciobarbus albanicus	Arachtos	Greece	1	La1
	Evinos		1	La2
	Pinios		1	La3
Luciobarbus mursa	Arax River	Armenia	1	*L.mursa*
L.cf.lorteti	Ceyhan River	Turkey	1	
Barbus petenyii	Ialomita River	Romania		

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GENERAL DISCUSSION

A summary of the principal results is here presented and a series of general issues will be discussed from phylogeography to biogeographical implications in different geographic scales of the evolution and diversifications patterns of the genus *Luciobarbus.*

The role of palaeogeography on the phylogeography of a *Luciobarbus* endemism from Iberian Peninsula

One of the works of the thesis was focused on *Luciobarbus sclateri* (Günther, 1868), a polytypic freshwater fish widely distributed in the mid-south basins of the Iberian Peninsula. Within the genus *Luciobarbus* this is the species that is the most widely distributed species in many independent basins, which are supposed to not have gene flow through a long time. Besides, the distribution range is located in an area of special complexity from a geological and paleohidrological point of view, which gives this species an interesting study model.

This work counted with the most thorough sampling made so far including all the small southern basins around the Gibraltar Strait that were here analyzed for the first time. In contrast to a previous work (Gante *et al.*, 2009) that highlighted the importance of Pleistocene climatic oscillations as main modulators of the evolutionary history of this Iberian endemism, we suggest a pre-Pleistocene scenario of paleodrainages rearrangements significantly influenced by tectonics, orogeny and river piracy that caused isolation, mixture and subsequently secondary contact between populations.

We found shared and private haplotypes between populations and we did not found a clear geographic differentiation of the populations of *L_.sclateri* as it has been pointed out for other cyprinids in the Iberian

Peninsula (Carmona *et al.*, 2000, Robalo *et al.*, 2007, Lopes-Cunha *et al.*, 2012, Perea *et al.,* 2016).

As for the time of inter population diversification, since Upper Miocene to Pleistocene three main cladogenetic events split the population of *L. sclateri* into three haplogroups: one distributed across all the basins, and two other groups, one exclusive from Segura Basin and the other one exclusive from some small southern basins of the Iberian Peninsula.

The first split, around 3 mya, is earlier than previously proposed (Gante *et al.*, 2009) but is in agreement with the emersion of the small basins located near the southwestern coastline during Middle Pliocene (Guerra-Merchan *et al.*, 2014). This period has been also considered important for the diversification of other freshwater fishes of the Iberian Peninsula, such as *Squalius* and *Chondrostoma* (Durand *et al.*, 2003, Doadrio and Carmona, 2004, Robalo *et al.*, 2007, Perea *et al.*, 2015). The second split promoted the separation of the exclusive haplotype of Segura in the Betic region from the rest of the populations in late Pliocene (around 2.2 mya). This area underwent important paleogeographical changes due to blocks rotation and the formation of large sedimentary in the area during Pliocene (Silva, 2014). The high number of haplotypes, both private and shared haplotypes, between populations led us to think of a scenario in which fluvial contacts and river piracy between basins are key factors to explain the structure of the population.

Besides, it is important to point out that also the species biology should be taken into account since barbels such as *L. sclateri* migrate upstream during the spawning period, a characteristic that could enable the rapid colonization of new basins when basins contact occurred. In opposite to barbel populations, the sedentary species of the genus *Squalius* sharing the same distribution range as *L. sclateri* show a clear genetic structure (Perea *et al.*, 2016).

Overall, it could be concluded that the patterns related to the geographical history of the hydrological basins seem to explain the patterns found in the diversification and population structure of *Luciobarbus sclateri*, as it has been suggested for other freshwater fishes but with a highest number of vicariant and dispersal events short that in other Iberian cyprinids to a short time period (Berendzen *et al.*, 2003, Lemmon *et al.* 2007, Kuchta *et al.*, 2016, Sousa-Santos *et al.*, 2016).

Comparison of evolutionary history and structure of allopatric populations

The Strait of Gibraltar is a key point in the geological and climatic history of the Mediterranean region and is an important area for Eurasian and African fauna exchange through time. For these reasons, the effects of distance and geographical barriers on the evolution and diversification patterns of sister taxa on both sides of the Strait of Gibraltar has been the focus of many studies (Rosenbaum *et al.*, 2002, Sanmartín, 2003, Bidegaray-Batista, 2011, Condamine *et al.*, 2013, Huesemann *et al.*, 2014, Novo *et al.*, 2015).

This part of the thesis focused on the implication of geological changes on the diversification patterns of the populations of *Luciobarbus* during Miocene, a period of multiple changes, ~~between~~ Iberia and Northern Africa. Mitochondrial and nuclear markers supported the phylogenetic relationship between some North African and Iberian populations. Previous studies based on mitochondrial markers and allozymes already pointed out that the so-called Iberian group was not monophyletic clustering the Iberian species of *Luciobarbus* with the North African *L. setivimensis* from the Soumman and Aissi rivers in Algeria (Doadrio *et al.*, 1998, Machordom and Doadrio, 2001). The connection between these two areas has been explained by the existence of the Betic–Rifian Massif including the Kabilian Mountains during most of the Paleogene ~~(Steininger and Rögl, 1984, López Martínez, 1989)~~ and it has been found for other organisms (Buckley *et al.*, 1996; García-París

et al., 1998; Álvarez *et al.*, 2000). In this thesis, we corroborate this mitochondrial clustering and found another species, recently described, from Moulouya Basin, *L. guercifensis* that is phylogenetically related the so-called Iberian group. In previous studies the common vicariant event explaining the difference between sisters groups of freshwater fish fauna is the opening of the Strait of Gibraltar at 5.3 mya (Doadrio, 1990, Machordom and Doadrio, 2001, Perdices and Doadrio, 2001), but our results indicated that an additional event happened before that time that could be claimed for the split between Iberian and North African populations during the Messinian, which would correspond to the Messinian gateways that were the last connection between Atlantic Ocean and Mediterranean Sea. The present-day distribution of *L. setivimensis* and *L. guercifensis* and its relation with the Iberian species could be explained by the block-rotations and reorganization that affected the area within the Gibraltar Arc systems from Tortonian to Present (Crespo-Blanc, 2016). As for the phylogenetic information rendered by the nuclear marker, the clustering of nuclear genome of the Algerian *L. sp* of the Chiffa basin and of *L. setivimensis* of the Setif basin could be due to secondary contacts between basins. Indeed, this is a common finding in European cyprinids which can present complete or partial presence of mtDNA of different species (Durand *et al.*, 2002, Perea *et al.*, 2016). We have identical nuclear alleles in some mitochondrial and morphological well-delimited species, which led us to think we are facing a case of nuclear ancestral polymorphism or that we are detecting a case of semipermeability of species-boundary, as it was already proposed for other Iberian species of *Luciobarbus* (Gante *et al.*, 2015). However this issue should be tested further once we are able to disentangle the polyploid puzzle that a nuclear approach has when dealing with polyploid organisms. Overall, the use of nuclear *actinII* proved to render a good phylogeographic structure when distinguishing between mitochondrial well-differentiated species, although it shows limitations for intra-species approaches, since there are alleles shared between populations, and phylogenetic approaches, since it could lead to polytomies and not so strongly supported phylogenies.

Due to the lack of resolution of nuclear genes for population studies recently we have tested microsatellites (up to 40), but they are still under study (not yet published) but as a results of this approach we have done the complete *L. rifensis* mitogenome (Corona-Santiago, 2017).

From Darwin to Mayr and so on, it is been considered that evolutionary diversification resulted almost entirely through the absence of gene flow (i.e: allopatric divergence) (Arnold, 2006). For a long time it is been assumed that genetic exchange between species is, by definition, impossible and methodologies delimiting species have been constrained by reproductive isolation concept (Dobzhansky, 1935, 1937, Sites and Marshall 2004). Nowadays, DNA sequencing and molecular works have shown that invasions of genome are widespread, with potentially important consequences in speciation, diversity and evolutionary biology (Mallet, 2005). The existence of gene flow between two well-differentiated species was thought to be common in plants and organisms such as amphibians but with the advent of molecular works is more common in vertebrates than previously described thought. For instance, nuclear DNA shared between two individuals belonging to different species of whales (Glober *et al.*, 2010). In *Luciobarbus* gene semipermiability has been described and speciation with flow could be played a role more important than previously thought, which makes this an interesting future line of work.

Phylogenetic relationships and biogeographical implications

One of the objectives of this thesis was to infer the evolutionary history and the phylogenetic relationships of the species comprising the genus *Luciobarbus* Heckel, 1843, ~~in~~ western Palearctic counting with the most complete phylogeny of the genus with the incorporation of ~~five~~ new described species and a nuclear marker. In the light of our results, the genus *Luciobarbus* is divided into two main lineages, one constituted by *L. mursa* and a second one comprised by the rest of the species. *Luciobarbus mursa,* is distributed in central Asia and Iran and its basal position to the rest of the

species of *Luciobarbus,* was previously observed by Levin *et al.*, (2012). The second lineage of *Luciobarbus* is comprised of three clades: clade I, constituted by *L._albanicus,* clade II, the so-called Iberian clade (here Moroccan *L. guercifensis* and Algerian *L.setivimensis,* are included) and clade III, comprised by Eastern and North African species. In contrast to previous studies we resolved basal nodes that remained unambiguous and that were interpreted as rapid radiation through Lago mare Phase during the Messinian (Tsinegopopulous *et al.*, 2003, Levin *et al.*, 2012, Geiger *et al.*, 2014). The results indicate a basal split in the Middle Miocene, an important period for the Mediterranean area when the Paratethys began to divide in different isolated basins. From this point on the genus underwent different diversification events linked to geological changes affecting circum-Mediterranean landmasses. On one hand, our results support the dispersion during the Middle Miocene through the Arabian Plateau of the species of *Luciobarbus* currently inhabiting North Africa, when sea regression allowed a connection through the Arabian Plateau between Eurasia and Africa (Meleunkamp *et al.* 2003). This is agreement with what has been postulated for other cyprinids (Otero *et al.*, 2001, Perea *et al.*, 2010). On the other hand, the presence of fossils of *Luciobarbus* in central Europe dated around 11 mya (Böhme, 2002) suggests that *Luciobarbus* could have also been present in Central Europe through the ancient Danube and posteriorly in the Iberian Peninsula. A change on environmental conditions could be claimed as factor of population extinction and displacement that led to its present-day absence of *Luciobarbus* in Central Europe. The evolution of landmasses and its influence on freshwater network has a great imprint on the diversification patterns of organisms such as primary freshwater fishes. In the case of *Luciobarbus* we have found an evolutionary pattern more linked to geological rather than to climatic changes, even though we speculate that the absence of *Luciobarbus* population in Central Europe nowadays could also be due to orogeny uplift and to a shift in the environmental conditions which did not fit the ecological requirements of this genus.

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